The construction and analysis of low TCF11/Nrf1 expressing plasmids - regarding transactivational ability and intracellular localization

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Cand. Scient. thesis in biotechnology 2003

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Acknowledgement

The work presented was completed at the Biotechnology Center in Oslo, at the University of Oslo, in the period from August 2001 to December 2003.

Prof. Anne-Brit Kolstø has been my main supervisor. I am thankful for her guidance, patience, and for providing me with excellent working facilities throughout the research period.

I am extremely grateful to Cathrine Husberg who, beyond the call of duty, has taken her time to guide and encourage me, and for her top-notch feedback throughout the study!

A big thumbs up to the other members of the group as well, Elisa Bjørgo and Jeanette Aarem, for their positivism and helpfulness. They have, alongside the people at UiO, created a stimulating and enjoyable working environment.

Thanks also to my supervisor at UiO, Prof. William Davis, who has kindly taken care of the administrative tasks. His critique and questions on the written work were also greatly appreciated.

Finally, I'm indebted to my better half Jannicke and my family for their unconditional support and confidence in me.

Oslo, December 2003

Lars-Egil Fallang

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Abbreviations

12S E1A	243 residue Adenovirus type 5 early region 1A
aa	Amino acid
Ab	Antibody
AD	Activating domain
AFX	Acute lymphoblastic leukemia 1 fused gene from chromosome X
AMP	Adenosine 5'-monophosphate
Amp	Ampicillin
AP	Alkaline phosphatase
Ap-1	Activator protein-1
APS	Ammonium-persulfat
ARE	Antioxidant response element
ARP-1	Apolipoprotein A1 regulatory protein-1
ATF4	Activation transcription factor 4
ATP	Adenosine 5'-triphosphate
Bach1, 2	BTB and CNC homology factor 1, 2
bp	base pair
Brn-2	Brain-2 transcription factor
BSA	Bovine serum albumin
BTB	Broad complex tramtrack bric-a-brac
bZIP	Basic-region leucine zipper
C/EBP	CCAAT/enhancer-binding protein
CBP	CREB-binding protein
CMV	Cytomegalovirus (immediate-early)
CNC	Cap'n'collar
COUP-TFI	Chicken ovalbumin upstream promoter-transcription factor 1
CREB	cAMP-responsive element binding protein
CREM	cAMP-responsive element modulator
Crm1	Chromosome region maintenance 1
DHT	Dihydrotestosterone
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleosidtriphosphate
dsDNA	Double strand DNA
DTT	Dithiotreitol
EDTA	Ethylendiamintetra aceticacid
ECF	Enhanced chemifluorescence
EtBr	Ethidium Bromide
FITC	Fluorescein isothiocyanate
γ-GCS	γ-Glutamylcystein synthetase
GCN4	General control protein 4
GSS	Glutathione synthetase gene
GST	Glutathione S-transferase
GTF	Genetral transcription factor
HAT	Histone acetyl transferase
HCl	Hydrochloric acid
HDAC	Histone deacetylase
HepG2	Human hepatoma G2
HIV 1 LTR	Human Immunodeficiency Virus Type 1á Long Terminal Repeat
HL	Hodkin lymphoma
HNF-1, -3β, -4	Hepatocyte nuclear factor 1, 3β, 4
HO-1	Heme oxygenase-1
HRS	Hodkin and Reed/Sternberg
HSV-TK	Herpes simplex virus-thymidine kinase
HTH	Helix-turn-Helix
IgG	Immunoglobulin G
IκB	Inhibitor of κB
kb	kilo base
kDa	kilo Dalton
LB	Luria-Bertani
LCR	Locus control region
LCR-F1	LCR- Factor 1
LyF-1	Lymphoid transcription factor-1
Maf	Musculoaponeurotic fibrosarcoma
MARE	Maf recognition element
MK2	MAPKAP kinase 2
MQ-water	MilliQ-purified water
mRNA	messenger RNA
NE	Nuclear envelope
NES	Nuclear export signal
NF-κB	Nuclear factor κB
NG108	Neuroblastoma Glioma 108
NLS	Nuclear localization signal
NPC	Nuclear pore complex
NQO1	NAD(P)H:Quinone oxidoreductase 1
Nrf1, 2, 3	NF-E2 related factor 1, 2, 3
NURF	Nucleosome remodeling factor
ON	Over night
OD	Optical density
P/CAF	p300/CBP associated factor
p300	Adenovirus E1A-associated 300kD protein
PAGE	Polyacrylamide gel electrophoresis

PBGD	Porhobilinogen deaminase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PIC	Preinitiation complex
Pit-1	Pituitary specific factor 1
Pol II	Polymerase II
Poly(A)	Polyadenylation
PPARα	Peroxisome proliferator-activated receptor α
PVDF	Polyvinylidene difluoride
R2C	Rat testicular interstitial cells clone 2
Rluc	Renilla luciferase
RT	Room temperature
Saos-2	Human osteosarcoma 2
SDS	Sodium dodecyl sulfate
SWI/SNF	Switch/Sucrose Non-Fermentation genes
TAE-buffer	Tris acetic EDTA buffer
TAF	TBP-associated factor
Tax	Trans-activator/X Region
TBE-buffer	Tris Boric EDTA buffer
TBP	TATA-binding protein
TCF11	Transcription factor 11
TCF3	Transcription factor 3
TEMED	N, N, N', N'-tetramethylethylenediamine
TFIIA	Transcription factor II A
tk-CAT	Chloramphenicol acetyltransferase controlled by HSV-TK
Tris	Hydroxymethyl aminomethane
UV	Ultraviolet
v-HNF 1	Hepatocyte nuclear factor 1 beta
VP16	Virion protein 16 of herpes simplex virus
Yap1	Yeast AP-1 like transcription factor

Abstract

TCF11 is a regulatory transcription factor belonging to the CNC-bZIP family. The specific biological function of this protein is still unknown. However, knockout studies in mice have revealed its importance during embryo development, and other studies have also displayed its involvement in the cell's defense system against oxidants and carcinogens. The transactivating ability and intracellular localization of TCF11 and the isoform Nrf1 have been studied in cells using high expression plasmids. Due to the recent findings that over-expression of TCF11 in transfected cells caused an increase in cell mortality, the need for lower TCF11/Nrf1 expressing plasmids emerged. An additional reason for constructing the low expression plasmids was to study the localization and transactivating abilities of the proteins at levels closer to the endogenous situation. The transactivating ability was estimated by measuring the luciferase activity in COS-1 cells transiently co-transfected with a reporter plasmid and a high or low TCF11/Nrf1 expressing plasmid. The intracellular localization images were acquired by means of epifluorescence and confocal microscopy. The initial low expression constructs proved unsuitable due to the empty vector's ability to cause indirect activation of the reporter plasmid. The second set of constructs were low expression plasmids that permitted verification of the nuclear detainment of Nrf1. However, further intracellular compartmentalization could not be detected for either Nrf1 or TCF11. In addition, TCF11 displayed a higher transactivating ability compared to Nrf1.

1 Introduction

Inside the nucleus of virtually every cell in a eukaryotic organism is a complex set of hereditary instructions encoded in the DNA (deoxyribonucleic acid). Within the DNA are short segments called genes that are transcribed into RNA (ribonucleic acid), which in turn is translated into proteins. Proteins are macromolecules that act as the cell's building blocks and carry out most of the organism's cellular functions. The differentiation between cells in a multicellular organism is the result of selective expression of genes, allowing for the creation of highly specialized phenotypically distinct cells. This selectivity is generally accomplished by controlling the initiation and inhibition of DNA to RNA transcription, which makes this control system one of the most important mechanisms of cellular regulation. One group of proteins that are key factors in transcription regulation is known as the regulatory transcription factors. The TCF11 protein studied in this thesis belongs to this group.

1.1 Transcription initiation in eukaryotic cells

The transcription of protein-coding genes in multicellular organisms is a highly complex, multi-level process. In general, transcription is thought to be initiated through the binding of regulatory transcription factors to activating gene sequences. In turn, these activators recruit the chromatin modifying complexes and the transcription initiation apparatus, allowing for transcription elongation to begin (1). The intricacies of this process will be reviewed in more detail.

1.1.1 Chromatin modification

Inside the nucleus the DNA is arranged into chromatin, a nucleoprotein complex consisting of nucleosomes, or DNA tightly packed around a histone octamer. This configuration is essential for the compact packaging of the genome, but may at the same time repress the binding of transcription-associated factors to their respective target sequences, thereby obstructing transcription (1). However, several activating transcription factors that have the capability of recognizing upstream activating sequences within the chromatin can alleviate this repression (2). These factors possess

the ability to recruit protein complexes that alter the configuration of the chromatin. The altering complexes can be divided into two main classes: chromatin remodeling and chromatin modifying. The chromatin remodeling complexes, like SWI/SNF and NURF, remodel the chromatin by breaking and reforming the histone-DNA contacts in an ATP-dependent manner (3,4). The chromatin modifying complexes, on the other hand, can modify the histones through acetylation/deacetylation, phosphorylation, methylation and ubiquitination (1). As an example, the acetylation of histones disrupts the chromatin and is completed by histone acetyl transferases (HATs, e.g. CBP/p300 and P/CAF) (5,6). Deacetylation by means of histone deacetylases (HDACs, e.g. HDAC1), on the other hand, has the opposite effect (7). Both the remodeling and the modifying complexes have been shown to be crucial for transcription initiation (3).

1.1.2 The basal transcription machinery

Once the chromatin is disrupted, several activating transcription factors, like VP16 and p45 NF-E2 (8,9), have been shown to subsequently recruit and stabilize the transcription apparatus to the promoter (1). At the base of this basal transcription machinery termed the preinitiation complex (PIC), is an enzyme called RNA polymerase II (Pol II), one of the three known RNA polymerases (10). This mRNA-transcribing enzyme requires the recruitment of several additional factors in order for transcription to be initiated. Among the necessary factors are the general transcription factors (GTFs) TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH (11), which have been shown to; aid in the recruitment of RNA Pol II to the promoter, the melting of the DNA helix, and also to assist in the following elongation (12). There are two possible models explaining the manner in which the PIC assembles, the ordered assembly model and the holoenzyme model (Fig. 1-1). The ordered assembly was the initial model based on a sequential recruitment of the required factors. The assembly is initiated by the binding of GTF TFIID to the TATA box, an A/T-rich promoter element. TFIID is a multi-subunit factor composed of the TATA-binding protein (TBP) and the TBP-associated factors (TAFs). The TFIIA coactivator subsequently binds to the TFIID-DNA complex, which is stabilized by the binding of the TFIIB. The resulting complex acts as a platform for the recruitment of RNA Pol II and TFIIF. The PIC is completed by the GTFs TFIIE and TFIIH.

The more recent holoenzyme model, on the other hand, suggests the presence of a pre-assembled complex where several of the GTFs are associated with RNA Pol II (13,14). This model suggests that the formation of the TFIID-TFIIA-DNA complex is followed by the recruitment of the holoenzyme, thus forming the PIC. The transcription initiation, including the chromatin modification and the two models of PIC assembly, is shown in figure 1-1.

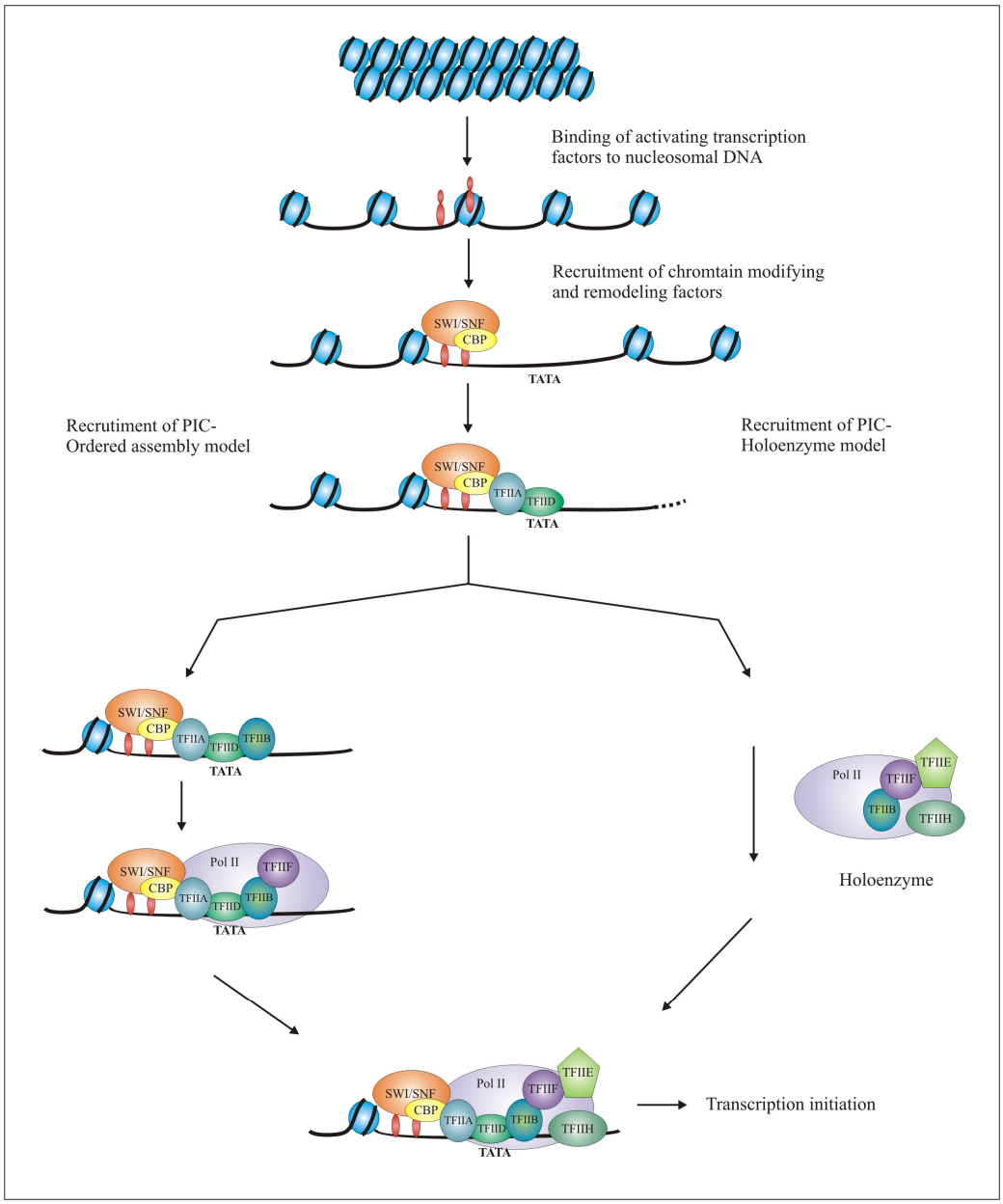

Figure 1-1 A schematic representation of transcription initiation. The representation includes the binding of transcription activators to the chromatin, the recruitment of the chromatin remodeling (e.g. SWI/SNF) and modifying (e.g. CBP) complexes, leading up to the two different models of PIC assembly. (This figure is modified from (13) and (15)).

1.1.3 Transcription elongation

Once the PIC is stably assembled on the promoter, elongation can be activated. The switch from initiation to elongation occurs through a number of events, including the melting of DNA to form an open complex, promoter clearance, and the disassociation of various factors from the PIC, thus turning it into an elongation enzyme (1,13). Regulatory transcription factors have been shown to be involved in the switch from initiation to elongation (16). As an example, the GTF TFIIF has been suggested to require the transcription factor c-Myc in order to stimulate the elongation process by lowering the frequency of abortive initiation (17). Another GTF, the TFIIH, aids in the conversion of PIC into an elongation enzyme through phosphorylation of the C-terminal tail of RNA Pol II. This process is thought to be mediated by the presence of TFIIE and the regulatory transcription factor GAL11 (18).

1.2 Regulatory transcription factors

The regulatory transcription factors modulate transcription of target genes by their binding to specific upstream regulating sequences. As mentioned earlier, these factors can affect several steps in the process of transcription, including chromatin remodeling, PIC formation, promoter clearance and elongation. The factors may not only aid in the activation of transcription, but some also repress it, while others can still do both. The factors normally have two functional domains: one DNA binding domain for recruitment to DNA, and one activating or repression domain for transcription regulation (19). The transcription factors are divided into families on the basis of their DNA-binding domains. The four most common are: 1) Helix-turn-helix, 2) zinc-coordinating, 3) helix-loop-helix and 4) leucine-zipper (20).

The activating domains (AD) are responsible for the interaction with factors of the transcription machinery and thereby aid in transcription activation. These domains are not as well defined as the DNA-binding domains because they lack a defined secondary structure in the absence of their target proteins. The ADs can normally be placed within one of the following groups: 1) Acidic, 2) glutamine-rich and 3)

proline-rich. However, not all ADs fit into these categories and several novel motifs have been suggested, such as the proposed leucine-rich acidic region (21).
Some regulatory transcription factors may also repress transcription in a gene-specific manner. Generally, these factors are thought to inhibit the activators from binding to the promoter, suppress the function of a promoter-bound activator, or hinder the assembly of the transcription machinery (13). However, the understanding of transcriptional repression is far less advanced compared to that of transcriptional activation.

1.2.1 bZIP transcription factors

The bZIP superfamily of transcription factors is a group of proteins found in virtually all eukaryotic organisms (22). These transcription factors are obligate homo- or heterodimers, and are characterized by having a basic-region leucine zipper domain (bZIP) located at the C-terminal end of a single α-helix. The bZIP domain contains two structural features, the dimerization and the DNA-binding domains. The dimerization domain consists of a heptad repeat of leucines, or other hydrophobic amino acids, which creates an amphipathic helix. Dimerization occurs by means of interactions between the hydrophobic residues of two monomers, creating a superimposing coiled-coil structure referred to as a zipper (see Fig. 1-2). The adhesion is further influenced by the electrostatic attraction and repulsion of polar residues flanking the hydrophobic interaction surface of the helixes (23).

The major function of dimerization is to bring together the DNA-binding regions of each monomer, allowing the resulting dimer to bind to the target sites. This is a result of the target sites being of a palindromic nature, requiring a dimeric binding domain where each monomer binds to their respective half site. The DNA-binding domain consists of basic amino acids immediately preceding the dimerization domain. Examples of the bZIP transcription factors are GCN4, c-Jun and c-Fos (23). Notably, dimers of the Jun and Fos gene family create a protein complex known as the activator protein-1 (AP-1) transcription factor. This complex, consisting of either Jun-Fos or Jun-Jun dimers, has been shown to play an important role in regulating cellular proliferation and differentiation (24).

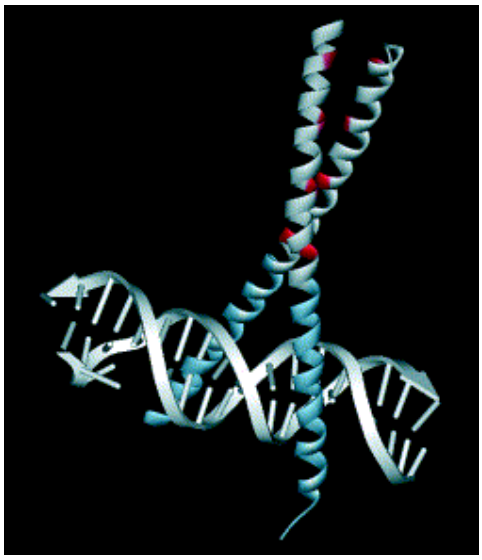

Figure 1-2 Illustration of a bZIP transcription factor (GCN4) bound to DNA. Leucine residues are marked in red. Illustration taken from (22).

1.2.2 CNC-bZIP

TCF11 belongs to a subset of the bZIP superfamily of proteins termed the CNC-bZIP transcription factors (25). This group of proteins, which also include p45 NF-E2, Nrf2, Nrf3, Bach1 and Bach2 (26), (27-29) share the features of the bZIP type proteins, but have an additional conserved structural domain, termed the Cap'n'collar (CNC)-domain. This domain was first noted in the *Drosophila Melanogaster* CNC gene, which is required for labial and mandibular development (30). The CNC domain immediately precedes the bZIP DNA-binding domain, but its function is unknown. In addition to containing the CNC sequence, members of this family show strong similarity in their basic DNA-binding domain, binding the NF-E2/AP-1 element with similar specificity. The consensus sequence is $^{T}/_{C}$GC<u>TGA$^{G}/_{C}$TCA</u>$^{T}/_{C}$, with the AP-1 site underlined. This DNA binding site is located in the promoter of numerous genes in the human genome, one being in the promoter regulating the erythroid-specific porphobilinogen deaminase (PBGD) gene (31). The transcription factors belonging to this family are presented below, with special reference to TCF11. A schematic representation of the factors in the CNC-bZIP family is shown in figure 1-3.

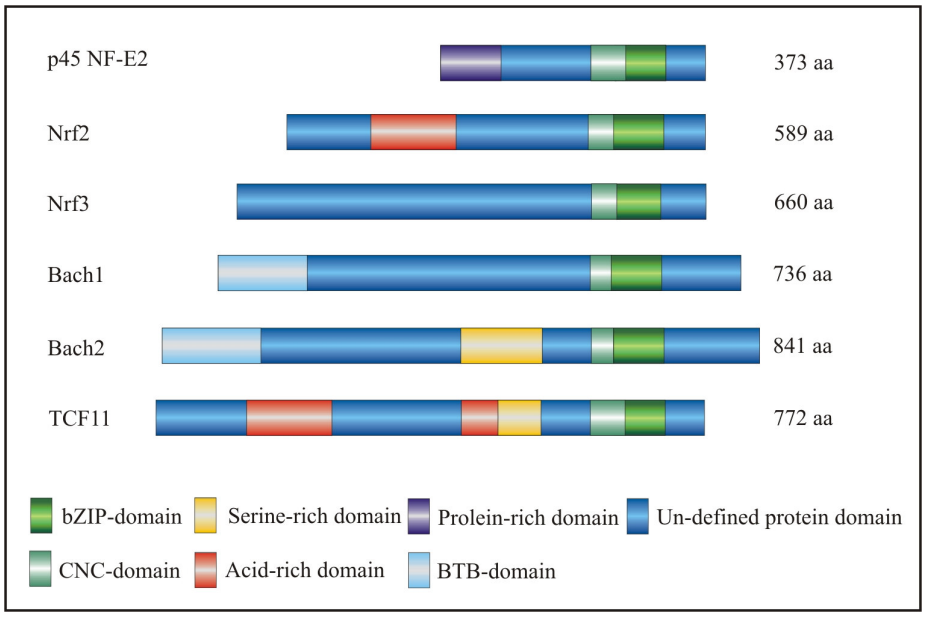

Figure 1-3 A schematic representation of the CNC-bZIP family of transcription factors. The domains and size expressed in amino acids (aa) are indicated.

1.2.2.1 p45 NF-E2

p45 NF-E2 is an erythroid-tissue specific transcription factor (32) that has a N-terminally proline-rich transactivation domain in addition to the CNC-bZIP domain (Fig. 1-3). This protein dimerizes with one of the three widely expressed small musculoaponeurotic fibrosarcoma proteins (Maf F, MafG or MafK) (33), forming the heterodimeric nuclear factor-erythroid 2 (NF-E2) complex. This dimer activates transcription by binding to the NF-E2/AP-1 site, and was originally identified as a complex activating PBGD and β-globin gene expression (31,34). The NF-E2 complex was therefore suggested to be a key regulatory element controlling pathways of heme and globin synthesis, leading to a balanced production of the hemoglobin components. However, a study showed that p45 NF-E2 knock-out mice were only slightly anemic, suggesting that a different bZIP factor could somewhat replace p45 NF-E2 in the NF-E2 complex (35). The same study reported a striking decrease in the platelet count, a finding that was later attributed to p45 NF-E2's involvement in the regulation of the thromboxane synthase protein, an enzyme essential for platelet formation and function (36). In addition, the activity of p45 NF-E2 has been shown to be elevated through interactions with co-factors like $TAF_{II}130$ and CBP (9,37). Furthermore, p45 NF-E2 possesses the ability to stimulate histone acetylation

mediated by CBP. This modification results in an increase in the ability to bind DNA and also an elevation of target gene expression (38).

1.2.2.2 Nrf2

Nrf2 is a ubiquitously expressed CNC-bZIP transcription factor having a N-terminally acidic transactivation domain (Fig. 1-3). Its protein sequence contains regions of high similarity to that of p45 NF-E2, and was therefore termed NF-E2 related factor 2 (Nrf2) (39). In addition to having similar DNA-binding abilities, Nrf2 has also been shown to dimerize with the small Maf proteins. However, in contrast to p45 NF-E2, the activity of Nrf2 decreased as a result of dimerization (40).

Nrf2 is linked to the regulation of detoxification and antioxidant proteins in cells. It appears as though Nrf2 is essential for the expression of phase two enzymes, which are necessary to produce antioxidants like glutathione (41). Nrf2 binds and activates through the antioxidant response elements (ARE), a binding site with strong sequence similarity to the NF-E2/AP-1 site. An ARE recognized by Nrf2 is located in the promoter of the γ-glutamylcysteine synthetase gene (γ-GCS), encoding for the rate-determining enzyme involved in the synthesis of glutathione (42).

1.2.2.3 Nrf3

The third NF-E2 related factor, Nrf3, was discovered in 1999 (28) (Fig. 1-3). This transcription factor has been detected in low levels in various tissues and has recently shown an elevated expression in Hodgkin and Reed/Sternberg (HRS) cells, which are unique to the Hodgkin lymphoma (HL) malignancy (43). Little is known about the function of Nrf3, but transient transfections have indicated that homodimers of the protein repress reporter induction (28). Nrf3 may also form a heterodimer with MafK, which appear to activate reporter induction through the binding of Maf recognition elements (MARE).

1.2.2.4 Bach1 and Bach2

Bach1 and Bach2 differ slightly from the other CNC-bZIP proteins in that they have an additional structure referred to as the "broad complex tramtrack bric-a-brac" (BTB) domain (29) (Fig. 1-3). This N-terminally located domain is found in a variety of DNA-binding proteins and has been shown to be of importance in transactivation and chromatin remodeling (44,45). Transcription factor Bach1 appears to be ubiquitously expressed, while Bach2 has only been detected in developing B-cells and neural cells (46). In addition, Bach2 expressed in neural cells appears to have an additional serine-rich domain of unknown function that is not present in B-cell-expressed Bach2. Like the p45 NF-E2 and Nrf proteins, these transcription factors can bind to the NF-E2/AP-1 site as either monomers or by forming dimers with the small Maf proteins. As an example, Bach1 dimerizing with MafK has been reported to repress transcription of the target genes (29). This heterodimer is associated with the regulation of heme oxygenase-1 (HO-1), a protein shown to protect cells from oxidative stress associated with high levels of heme. (47). The function of Bach2 is also stress-related and evidence has shown that its presence can induce apoptosis in response to oxidative stress (48).

1.2.2.5 TCF11

Transcription factor 11 (TCF11) is a ubiquitously expressed protein that was first identified by the Kolstø group in 1994 (25). This protein has three domains in addition to the CNC-bZIP domain, including an N-terminal acidic domain, an internal acidic domain, and an internal serine-rich domain (Fig. 1-3). The N-terminal acidic region has been found to function as TCF11's transactivation domain (49). For full transactivation to occur, however, the serine-rich domain has been shown to be essential, which is thought to be due to the several potential phosphorylation sites within this region. The internal acidic domain, however, has not yet been found to be crucial to the transactivating ability of the protein. Additional regions of importance in the TCF11 protein are the nuclear localization signal (NLS) located in the basic bZIP region, and the nuclear export signal (NES) located in the N-terminal transactivation domain (50).

Parallel to the discovery of TCF11, there were other research groups that isolated two additional protein isoforms of the same gene. TCF11 is therefore also referred to as Nrf1 (NF-E2 related factor 1 (51)) and LCR-F1 (Locus Control Region-Factor 1 (52)) (Fig. 1-4). The Nrf1 isoform is a result of alternative splicing (Section 1.3.1) within the gene, causing the loss of a 30 aa's segment in the N-terminal transactivation domain. The removed leucine rich sequence contains a NES-signal, and studies have indicated that Nrf1 therefore lacks the ability to be exported from the nucleus (50). Alternative splicing within the transactivation domain produces several other isoforms, ranging from 728 to 769 amino acid residues in size (25).

TCF11 isoforms are also created through alternative translation initiation (Section 1.3.1). Because the first initiation site in the TCF11 transcript is non-optimal, the translation is initiated from an internal initiation site. LCR-F1 is produced in this manner, resulting in a 447 aa's isoform lacking the N-terminal transactivation domain (25,52). As could be expected due to the absence of this domain, this isoform displays strict nuclear localization (50), and transient transfections have failed to detect any activity (49).

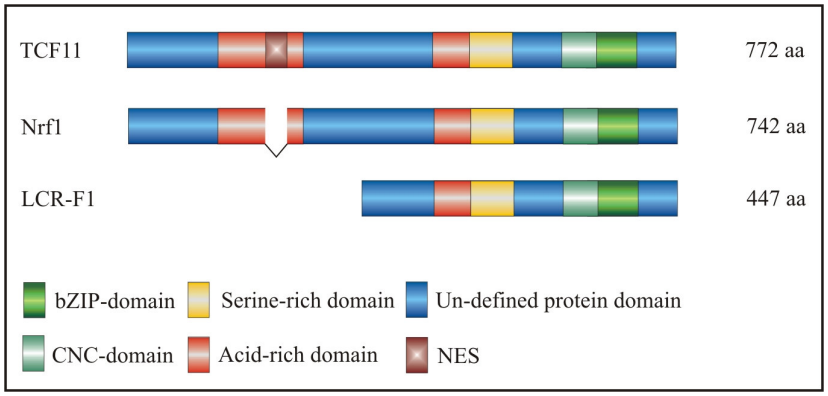

Figure 1-4 A schematic representation of TCF11, Nrf1 and LCR-F1. The domains, deletion and size expressed in aa's are indicated.

A common feature of the CNC-bZIP family of proteins appears to be the ability to dimerize with the small Maf proteins. TCF11 is no different, forming heterodimers with Maf that show higher affinity to the NF-E2/AP-1 site compared to the TCF11 homodimer (53). ATF4 (personal communication, A-B. Kolstø) and the Jun proteins (54) have also been suggested to be potential dimerization partners of TCF11.

As with all of the CNC-bZIP factors, studies have shown that TCF11 can bind and transactivate through the NF-E2/AP-1 site (55). However, TCF11 has not yet been detected to have a direct effect on the mRNA expression of genes under the control of this site (i.e. PBGD and β-globin). TCF11 has also been reported to activate through ARE-elements (56), as seen for Nrf2 (54,57). These NF-E2 like sequences are found in the promoter regions of genes associated with the cellular defense system against free radicals and toxic compounds. Two examples of these genes are the glutathione synthase (GSS) and the previously mentioned γ-GCS. These enzymes are necessary for the synthesis of glutathione, which is a ubiquitously expressed non-protein sulphydryl involved in the maintenance of the intracellular redox balance and elimination of free radicals (58). Studies have indicated that TCF11 plays an important role in the regulation of both enzymes, and subsequently the indirect regulation of glutathione (56). In addition, TCF11/Nrf1 is suggested to regulate the detoxifying enzymes NAD(P)H:quinone oxidoreductase (NQO1) and glutathione s-transferase (GST) (54,57). In view of these findings, TCF11 appears to be an essential factor in the cell's defense system against oxidants and carcinogens. Interestingly, recent studies present evidence that, although TCF11 and Nrf2 do not have identical functions in their response to oxidative stress, they have the ability to somewhat compensate for each other and coordinate their regulating ability (59).

TCF11 has also been found to be an essential protein in the embryo development. Studies on mouse embryos unable to express TCF11 proved to be fatal at an early stage. One study attributed the fatality to the lack of mesoderm formation, suggesting that TCF11 is essential for gastrulation (60). A different study found that the TCF11 deficiency caused anemia, and that TCF11 is involved in the early stages of red blood cell production (erythropoiesis) in the liver (61). Recent studies ascribed the reduced erythropoiesis and subsequent lethality to the hepatocytes being sensitized to oxidative stress as a result of the TCF11 deficiency (62). This is further evidence that TCF11 could be an essential factor in the redox balance in cells.

Notably, an increased mortality due to over-expression of TCF11 has been detected in both stably transfected HeLa cells and transiently transfected COS-1 cells (personal communication, C. Husberg). These observations present the possibility that TCF11 may be involved in the regulation of cell death.

1.3 Regulation of regulatory transcription factors

The level in which a target gene is transcribed is dependent both on the concentration and the activity of the regulatory transcription factors. The regulation of the transcription factors themselves is therefore a key control point of the target gene production. Several of the numerous mechanisms involved in the regulation of transcription factors are given below, with emphasis on the mechanisms affecting TCF11.

1.3.1 Regulation of the expression level

Regulation at the transcriptional level is the primary means by which the concentration of proteins is controlled. This allows the expression of transcription factors to be restricted to specific cells and tissues. An example of this is the regulatory transcription factor p45 NF-E2, which have been shown to be restricted to hematopoietic tissues and cell-lines (63). Another way in which the production of many cell-type specific transcription factors is regulated is by means of auto-regulation. This is a mechanism where factors, like Pit-1, are involved in their own transcriptional activation (64). TCF11, however, is a ubiquitously expressed protein and is not likely to be regulated by these mechanisms (25).

A different process in which the expressional level of a regulatory transcription factor is regulated is through alternative splicing of mRNA. Splicing is the process of excising the numerous non-coding introns from the coding exons in the pre-mRNAs (primary transcript), thereby creating functional mRNA. By splicing the exons in varied combinations, diverse mRNAs, and thereby proteins with different functions and activities can be produced from the same gene. One example is the CREM gene, which has been shown to generate both activators and repressors through alternative splicing (65). Alternative splicing can produce several isoforms of TCF11, one being the Nrf1 (Section 1.2.2.5). The activity level measurements of this isoform have given ambiguous results, however, displaying both an unchanged level of activity (49), and a reduced level of activity (personal communication, E. Bjørgo) when compared to TCF11.

A different manner in which various isoforms of the same transcription factor can be obtained is through initiation of translation from different start codons within the mRNA-transcript. While the optimal start codon sequence is called the Kozak-sequence (ACCAUGG), translation can also start from non-optimal sequences (66). The mRNA transcript of the transcription factor C/EBP gene contains both an early non-optimal and an internal perfect Kozak-sequence. In this case, the cell-regulated initiation from the distinct start sequences results in two protein isoforms with different transcription activation potentials, one being an activator (full-length form), while the other is a repressor (67). This mechanism has been shown to produce isoforms of TCF11, one being the LCR-F1 (Section 1.2.2.5). As mentioned earlier, this isoform has displayed a lack of activity on its own, and its presence has also been seen to reduce the transactivating ability of full-length TCF11 (56).

The stability, or rate of degradation, of an mRNA molecule is also a factor regulating the expression of transcription factors. Genes that are expressed in high levels, such as β-globin (68), requires mRNA's that are highly stable. This allows the protein translation to continue long after transcription is repressed. In contrast, numerous genes that code for transcription factors, such as c-Fos and c-Jun, have rapidly changing production levels and are therefore dependent on having mRNAs that are unstable and with short half-lives (69). The stability of mRNA is dependent on specific structural components, some of which include the 5'-cap structure and the 3'-polyadenylate (poly(A)) tail. These are key components in the major degradation pathway, which starts by the shortening of the poly(A) tail through exonuclease activity, followed by the removal of the 5'-cap, and the eventual bidirectional degradation of the mRNA (68). The rate of mRNA degradation can therefore be affected by the stability of these components. As an example, the stability of the HNF-1 protein is affected by variation in length of the poly(A) tail due to alternative splicing (70). Specific sequences in the molecule may also contribute to mRNA instability. One example is the AUUUA-sequence that appears to make mRNA unstable when occurring in multiple copies in the 3' untranslated region of the molecule (69,71). Studies have revealed a slightly larger isoform of TCF11 containing both a longer poly(A) tail and the presence of additional AU-rich sequences. Interestingly, the longer form appeared to be more abundant in all tissues examined (25).

1.3.2 Regulation of activation

Once the transcription factor has been translated from mRNA, the regulation is accomplished by controlling the activity level of the factor, thereby affecting the expression of target genes. Several of these regulating mechanisms are presented below.

1.3.2.1 Regulation of DNA-binding and transactivation

Post-translational modification of proteins is a way of achieving this kind of regulation. Phosphorylation and dephosphorylation of proteins are common modifications that either negatively or positively regulate transcription factor activity. In the case of the transcription regulator termed Snail, phosphorylation caused a reduction in the ability to activate transcription, while the DNA-binding ability was left intact (72). Phosphorylation has the opposite effect on CREB and increases the protein's transactivating ability by allowing for the interaction with co-factors CBP/p300 (73). The role of phosphorylation in the regulation of TCF11 has not been studied extensively. However, the multiple phosphorylation-sites observed in the serine-rich domain have been suggested to play an important role in regulating the activity of TCF11 (49).

The activity of a transcription factor can also be regulated through interactions with other proteins. Such interactions may modulate the DNA binding specificity of a protein, in addition to altering its potential as an activator or repressor of transcription. The bZIP-superfamily (Section 1.2.1) is an example of proteins that utilize this type of regulation. These proteins bind by means of their leucine zipper, forming homodimers and/or heterodimers with varying effect on the activity. As mentioned earlier, the heterodimeric complex consisting of p45 NF-E2 (Section 1.2.2.1) and a small Maf protein results in a transcriptional activating dimer, while homodimers of the small Maf proteins themselves are unable to activate transcription (74,75). TCF11 have also been observed to form dimers with the small Maf proteins (53). However, most of the heterodimers tested have displayed a repressed activity when compared to that of the TCF11 homodimer.

1.3.2.2 Regulation of intracellular localization

Restricted intracellular localization is another means of regulating the activity level of a regulatory transcription factor. Since the target DNA is located in the nucleus, restricting the protein's nucleocytoplasmic transportation can be an efficient way to control its activity. The nucleocytoplasmic transport occurs through the nuclear pore complexes (NPC), which penetrate the nuclear envelope surrounding the nucleus (76). The NPCs allow slow passive diffusion of proteins smaller than ~60 kDa, while larger molecules, like transcription factors, are dependent on active transportation (77). Active transportation in either direction across the nuclear envelope involves sequential steps. These include: recognition of the protein's transport signal by a transport receptor, docking of the protein/receptor complex at the NPC, translocation through the NPC and release of the transported protein (77,78). There are two different types of translocation signals depending on the direction of transport. Two well-studied examples are the previously mentioned NLS, which is essential for nuclear import (79), and NES, required for nuclear export (80,81).

One way to control the intracellular localization, and consequently the activating ability of a transcription factor, is therefore to regulate its affinity to the transport receptor by masking or unmasking the transport signal. This can be achieved either by phosphorylation or by association with accessory proteins (82). In the case of Smad4, phosphorylation caused the masking of NES and unmasking of NLS, resulting in nuclear localization and a subsequent increase in activation (83). However, phosphorylation may also decrease the activity by instigating cytoplasmic localization, either due to the unmasking of NES, as was the case for MK2 (84), or the masking of NLS, as seen for AFX. (85). The NF-κB, on the other hand, displayed cytoplasmic localization upon forming a complex with IκB (86). The IκB is an inhibitory protein that masks the NLS signal in the NF-κB protein, thereby rendering the protein inactive in the cytoplasm.

Some proteins also use nuclear shuttling to regulate their activity. These proteins continuously move between the cytoplasm and the nucleus, and their steady-state localization reflects their relative rates of nuclear import and export. An example of this is the yeast AP-1 like (Yap1) transcription factor. The steady state of this protein

is cytoplasmic, but shows nuclear accumulation when exposed to oxidative stress. This response is due to inhibition of the nuclear export pathway (87).

Unlike TCF11, Nrf1 lacks the functional NES signal and appears to be restricted to the nucleus. Interestingly, however, the nuclear accumulation of Nrf1 does not give an elevated activity compared to TCF11, as mentioned earlier.

1.3.2.3 Regulation of protein stability

Another means to terminate the activity of a transcription factor is by regulating the rate of protein degradation. By altering the protein stability, the transcription factor's longevity varies, thereby changing the overall activity of the protein. Examples of proteins regulated in this manner are the transcription regulators p53 and GCN4p, which are degraded by the well-documented ubiquitin-dependent proteolysis pathway (88,89). By conjugating with ubiquitin molecules, the proteins are tagged for the subsequent destruction by the proteasome (88). The role of the degradation pathway in regulating the activity of TCF11 is unknown.

The activity of a transcription factor will often consist of a compilation of the regulating mechanisms mentioned. In the case of transcription factor Pho4, multiple phosphorylation sites provide numerous layers of regulation that modulate its activity (90). For instance, both the nuclear export and import have seen to be mediated by phosphorylation. In addition, phosphorylation allows for the dimerization of Pho4 with the Pho2 transcription factor, needed for the transcriptional activation of the target gene PHO5.

1.4 Aims of the study

The transactivating ability and intracellular location of TCF11/Nrf1 have so far been studied in cell-lines by means of a high expression vector system. Due to the detected increase in cell mortality as a result of over-expression of TCF11/Nrf1, the aim of this study was to construct vectors that expressed TCF11 and Nrf1 at lower levels. Subsequently, the high and low expressing vectors would be compared with respect to the transactivating ability and intracellular localization of TCF11 and Nrf1.

2 Materials and Methods

Buffers and standard solutions were made according to Sambrook, *et al* (91) unless provided in the kits used.

2.1 Biological material

2.1.1 Growth of *E.coli*

Escherichea coli (*E.coli)* XL1-blue Mrf was grown in LB media (1% Trypton, 5.6 mM Glucose, 171 mM NaCl, 0.5% Yeast extract) containing 50 μg/ml ampicillin. Incubations were done in a Controlled Environment incubator shaker (New Brunswick Scientific) at 37°C over night (ON).

For screening purposes, the *E.coli* bacteria were plated on LB agar (LB, 1.25% Agar No.1) containing 50 μg/ml ampicillin, and incubated ON in a Termaks incubator. Other media used were SOB (2% Trypton, 0.5% Yeast extract, 9 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$) and SOC (2% Trypton, 0.5% Yeast extract, 20 mM Glucose, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$).

2.1.2 Growth of COS-1

COS-1 cells were cultured in Dulbecco's Modified Eagle's Medium with 1g/l glucose (DMEM, Life Technologies) added 10 % Foetal Calf Serum, 2 mM L-Glutamine, and 50 U/ml penicillin/Streptomycin. The incubations were done in a water-jacketed incubator (Forma Scientific) at 37°C and 5% CO_2.

2.1.3 Plasmid constructs

A list of pre-made plasmid constructs used in this study is displayed in table 2-1.

Table 2-1Pre-made plasmids used in this study

Name of plasmid	Description	Source
pcDNA3	Vector containing the strong promoter *human cytomegalovirus immediate-early (CMV)*, and is designed for high-level stable and transient expression in mammalian cells. The vector contains the ampicillin resistance gene as a selection marker.	Invitrogen
pcNrf1 (pcTCF11 Δ242-271)	The gene for full length TCF11, with the amino acid residues 242-271 excised, cloned into the pcDNA3 vector (Invitrogen).	Constructed by Dr. Elisa Bjørgo (50).
pcTCF11	The gene for full length TCF11 cloned into the pcDNA3 vector (Invitrogen).	Constructed by Dr. Cathrine Husberg (56).
3.2PBGDLuc	A pGL3-Enhancer Vector (Promega) containing the luciferase gene under the control of a part of the porphobilinogen deaminase (PBGD) gene promotor. The promoter region contains a single NF-E2 site which can be activated by TCF11. The reporter vector also contains the gene for ampicillin resistance as a selection marker.	Constructed by Dr. Paula Murphy (55).
3.2PBGDLuc Mut. 1	3.2PBGDLuc vector containing a one-base mutation in the NF-E2 binding site. This mutation is located outside the AP-1 binding site.	Constructed by Dr. Paula Murphy (55).
3.2PBGDLuc Mut. 2	3.2PBGDLuc vector containing a four-base mutation in the NF-E2 binding site. This mutation is located inside the AP-1 binding site.	Constructed by Dr. Paula Murphy (55).
pRL-TK	Vector containing the weak promoter *herpes simplex virus thymidine kinase (HSV-TK)*, with the ampicillin resistance gene as a selection marker.	Promega
puC19	Vector used for testing competent cells. The vector contains the gene for ampicillin resistance as a selection marker	Stratagene

2.2 Cloning and DNA techniques

2.2.1 Isolation of plasmid-DNA from E.coli

Plasmid DNA isolation from ON LB cultures of *E.coli* was done by alkaline lysis of the bacterial cell wall in the presence of SDS detergent. This treatment opens the cell wall, allowing for denaturing of the genomic DNA and proteins, while releasing the plasmid DNA. The plasmid DNA was precipitated in isopropanol and finally dissolved in MilliQ-purified water (MQ-water).

Two types of DNA isolations were used depending on the purpose, amount, and purity of the DNA needed. For the isolation of small quantities of plasmid DNA used for screening purposes, the minipreparation protocol described in Sambrook, *et al* (91) was followed. For the isolation of larger quantities of purified DNA, the Plasmid Midi Kit (Qiagen) was used, as described by the manufacturer. A protocol deviation was the replacing of the supplied TE-buffer with MQ-water. The midiprepared plasmid DNA was quantified (Section 2.2.3).

2.2.2 Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to separate, identify and purify DNA molecules. The molecules are separated by means of an electrical field, where the rate of movement is dependent on the voltage applied, the ionic strength of the buffer used, and the concentration of the agarose gel. The intercalating agent ethidium bromide (EtBr) was added to the gel (0.5µg/ml) to allow for UV visualization by means of a Saveen TFP-35M (Vilber Lourmat) apparatus. The Power Pac300 (BioRad) was used as the power source. Two types of agarose gel electrophoresis were used, analytical and preparative.

Analytical agarose gel electrophoresis was used to quantify DNA and to determine the size of DNA molecules. The gels ranged from 0.8-2.0% agarose (Sigma) using 1xTBE as a running buffer.

Preparative Agarose Gel Electrophoresis was used to isolate purified DNA from 1.0% agarose gels (SeaKem® GTG® agarose [FMC BioProducts]). The running buffer used with this

agarose was 1xTAE. DNA fragments to be purified were extracted from agarose according to the QIAEX II Gel Extraction Kit (QIAGEN) protocol.

2.2.3 Quantification of DNA

DNA was quantified primarily by means of the GeneQuant (Pharmacia Biotech) spectrophotometer. The concentration of DNA in a sample was determined by measuring the optical density (OD) at 260nm, where a value of 1 corresponds to a concentration of 50 μg/ml for dsDNA and 30 μg/ml for ssDNA.

In addition, some DNA concentrations were determined through analytical agarose gel electrophoresis (Section 2.2.2). Here, quantification was done by comparing DNA fragment intensity to that of known concentrations of a DNA standard (Lambda DNA/*Hind* III [Promega]).

2.2.4 Digestion of DNA with restriction enzymes

Digestion of DNA was performed to do restriction mapping and for constructing plasmids. The restriction enzymes, with their accompanying buffers (NEB), were used as recommended by the manufacturer. All reactions were incubated in a PTC-100™ (MJ Research, Inc) PCR apparatus, while heat inactivation was completed in a BBD2 (Grant-Boekel) heating block.

2.2.5 Ligation of DNA fragments

Ligation was performed using T4 DNA ligase in ligation buffer (NEB) as recommended by the manufacturer. All reactions were incubated in a PTC-100™ (MJ Research, Inc) PCR apparatus ON at 16˚C.

2.2.6 Blunting of DNA

Klenow DNA Polymerase (NEB) with added dNTPs (Amersham Pharmacia Biotech) was used to blunt DNA fragments with sticky ends by means of its 5'→3' polymerase activity. The reaction was set up as recommended by the manufacturer. A PTC-100™ (MJ Research, Inc) PCR apparatus was used for the 15 minute incubation at 25˚C, while a BBD2 (Grant-Boekel) heating block was used for the 10 minute heat inactivation at 75˚C.

2.2.7 Making electrocompetent cells

In order to make *E.coli* competent, or susceptical to the uptake of foreign DNA, the bacterial outer and inner cell membrane will have to be made penetrable. This was achieved by exposing *E.coli* to a combination of heat shocking and DMSO, as described in Sambrook et al. (91). The cells were frozen in liquid nitrogen and kept at -80°C.

2.2.8 Transformation of *E.coli* by electroporation

Electrocompetent *E.coli* (Section 2.2.7) were transformed using electroporation. By means of electric pulses, generated by the Gene Pulser/Pulse Controller (BioRad) apparatus, transient pores are created in the membranes of the *E.coli*, allowing for DNA uptake.

1 ng of plasmid DNA was added to 40 µl of thawed electrocompetent cells and incubated for 1 minute on ice. The suspension was transferred to a cold cuvette (BioRad) and electroporated with a pulse of 2.5 kV, resistance of 400Ω, and a capacitance of 25 µF. SOC-media at 37°C was immediately added, and the suspension was incubated on a shaker at 37°C for 1 hour. The cell suspension was plated on LB agar plates containing 50 mg/ml ampicillin and incubated at 37°C ON in order to screen for transformed cells. The transformation efficiency of the electrocompetent cells was determined by transforming the competent cells with a pUC19 control plasmid. By plating a dilution series of the transformed suspension, the efficiency was calculated as the number of colonies per µg of pUC19.

2.2.9 Screening for transformants

Screening for transformants was done in two steps. Plasmid DNA was miniprepared from ON cultures of transformed bacterial colonies, followed by a restriction analysis to screen for desired clones. The plasmid DNA was also sequenced (Section 2.2.10) to further verify the obtained clone.

2.2.10 Automatic sequencing of DNA

The automatic sequencing was completed by Henning A. Johansen at the Biotechnology centre of Oslo. The sequencing was done by using an A.L.F. DNA Sequencer™ (Pharmacia Biotech). The sequencing primer (5' GAAACGGAGCAGGGATTCGG 3') was synthesized by Dr. Eshrat Babie at the DNA synthesis laboratory in the Biotechnology centre of Oslo.

2.3 Cell Techniques

2.3.1 Transfection

Transfection is the process in which foreign DNA is taken up by eukaryotic cells and is essential in studying gene function and regulation. The uptake of DNA is temporary in a transient transfection, while in a stable transfection the DNA is incorporated into the cell's genome. In this study, transient transfection was done by using FuGENE (Roche Molecular Biochemicals) as a transfection reagent. FuGENE consists of a lipid mixture that encapsulates the DNA and transports it into the cell through cell membrane fusion. However, the details of this mechanism are not known.

COS-1 cells (70-80% confluent) were split 18-20 hours before transfection, allowing the cells to reach 50-60% confluence. The cells were transfected with 0.8-2.0 µg DNA using a FuGENE to DNA ratio of 3 µl :1 µg. The manufacturer's protocol was used.

2.3.2 Luciferase-assay

The luciferase-assay was used to study TCF11's ability to activate transcription. The assay is based on the ability of TCF11 to bind to its respective binding sites and activate transcription of the luciferase gene located in the PBGD reporter plasmid. Luciferase, in turn, reacts with and breaks down added luciferin, causing the emition of a yellow-green light (Fig. 2-1). This light is detected and quantized by a luminometer. The light intensity can therefore be used to correlate TCF11's ability to activate transcription.

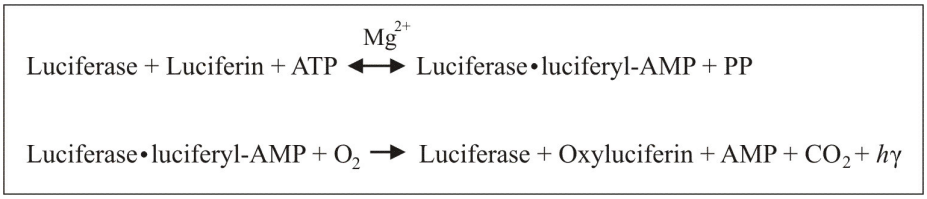

Figure 2-1The catalyzed reaction of the luciferase enzyme. The enzyme reacts with luciferin and as a result, light (*hγ)* is emitted.

2.3.2.1 Luciferase activity measurement

Three parallels of each DNA construct, along with a standard amount of 0.2µg of the PBGD reporter plasmid, were transfected into COS-1 cells and incubated for 48 hours before harvesting. All samples were kept on ice at all times.

Growth media was removed and the cells were washed twice in PBS. 150 µl of luciferase-lysis buffer (50 mM Tris-MES [pH 7.8], 1mM DTT, 0.1% Triton X-100), was added and incubated for 5 minutes. The cells were harvested and centrifuged at 13000 rpm for 5 minutes at 4°C (Biofuge fresco, Heraeus instruments). 5, 10 and 20 µl of the supernatant was mixed with 195, 190 and 180 µl luciferase cocktail (10 mM $Mg(OAc)_2$, 50mM Tris-MES [pH 7.8], 2mM ATP) respectively, and the luciferase activity was measured using a MicroLumat Plus luminometer (EG&G Berthold).

2.3.2.2 Normalization of the luciferase activity

The relative protein content in the cell extracts was used to normalize the luciferase activity measurements. A Protein assay kit (BioRad) was used for this purpose.

The concentrated Bio-Rad protein assay substrate was diluted four times with MQ-water. 2 ml of the diluted substrate and 5-10 µl of each sample was added to disposable cuvettes (Rankell), followed by a 5-minute incubation. The samples' absorbance of light at 595 nm were measured by using the U-1500 Spectrophotometer (Hitatchi Instruments). The blank consisted of substrate lacking sample.

2.3.3 Western Analysis

Western analysis was used to detect and quantify specific proteins in transfected cells, and involves SDS-PAGE and Western blotting.

2.3.3.1 Harvesting of cells

Two parallels of each DNA construct were transfected into COS-1 cells and incubated for 24-30 hours before harvesting.

Growth media was removed and the cells were washed twice with PBS. 75 µl of 2 x SDS gel loading buffer (100mM Tris-HCl [pH 6.8], 200mM DTT, 4% SDS, 0.1% Bromphenol blue, 20% Glycerol) heated to 85°C was added to the cells. The cells were harvested, denatured at 100°C for 10 minutes and filtered through a 25GA needle (Becton Dickinson). Following a centrifugation (Biofuge fresco, Heraeus instruments) at 13000 rpm for 5 minutes, the supernatant was applied to an SDS-PAGE-gel.

2.3.3.2 SDS-polyacrylamide gel electrophoresis

SDS-PAGE was used in the size separation of proteins from lysed cells. The negatively charged detergent sodium dodecyl sulfate (SDS) binds and denatures proteins, thus masking the charge of the proteins. The proteins can therefore be separated based solely on size and not charge. A concentrating gel (”stacking gel”) was applied on top of the resolving gel in order to concentrate the proteins. In this work, the electrophoresis equipment Mini Protean II (BioRad) and Power Pac 300 (BioRad) were used.

A 10% resolving gel was made (1.7 ml 29% Acrylamide/1% Bisacrylamide, 1.3 ml 1.5M Tris [pH 8.8], 0.05 ml 10% SDS, 1.9 ml MQ-water, 25 µl 10% APS, 2.5 µl TEMED) and poured between the glass plates, allowing for a 30 minute polymerization. Subsequently, a 5% stacking gel solution (0.33 ml 29% Acrylamide/1% Bisacrylamide, 0.25 ml 1.5M Tris [pH 6.8], 0.02 10% SDS, 1.4 ml MQ-water, 10 µl 10% APS, 2 µl TEMED) was poured onto the resolving gel and the comb was inserted.

After polymerization, the comb was removed and the wells were washed with MQ-water to remove unpolymerized acrylamide. The gel was placed in the electrophoresis apparatus and filled with 1x SDS-electrophoresis buffer (125 mM Tris, 1 M Glycine, 0.5% SDS). The protein samples were thawed, denatured at 95°C for 5 minutes, and loaded on the gel in 15-20 µl aliquots. 10 µl of the Prestained Broad Range marker (BioRad) was used as a size marker. The samples were run at 100V through the stacking gel and 120-150V through the resolving gel (~2 hours).

2.3.3.3 Western blotting

Western blotting was used to detect a particular protein(s) in a gel using antibodies. Proteins separated on an SDS-PAGE were transferred to a PVDF membrane (Immobilon-P, Millipore) by means of an electric current. Following the transfer, the membrane was incubated with a primary antibody that is binding-specific for the desired protein. By incubating the membrane with an alkaline phosphatase (AP)-conjugated secondary antibody that has a high binding specificity for the primary antibody, the proteins can be visualized by adding AP

dephosphorylating ECF™ Substrate (Bio-Rad), which produces a fluorescent product. The Trans-Blot Cell (BioRad) blotting apparatus and the Microcomputer Electrophoresis Power Supply (CONSORT) were used.

A PVDF membrane was activated in methanol for 1 minute and washed with ice-cold blotting buffer (25 mM Tris, 200mM Glycine, 20% Methanol). The transfer unit was assembled (Fig. 2-2) and placed in the blotting chamber, which contained blotting buffer and a cooling element, with the membrane towards the anode and the SDS-gel towards the cathode. The blotting proceeded for 2 hours at 85V, replacing the cooling element after 1 hour. Subsequently, the membrane was blocked in blocking solution (5% Dry milk, 1% Glycine, in TBST (165 mM NaCl, 10 mM Tris [pH 8.0], 0.1% Tween-20)) for 1 hour at room temperature (RT), washed three times in TBST at 4°C, followed by primary antibody incubation (1:1000 of rabbit anti-Nrf1 (C-19) [Santa Cruz Biotechnology] in TBST and 5% dry milk) ON at 4°C. After completed incubation, the membrane was washed three times in TBST, followed by a 2 hour incubation with the secondary antibody (1:2000 Donkey anti-rabbit AP conjugate [Santa Cruz Biotechnology] in TBST and 5% dry milk) at 4°C. The membrane was washed three times in TBST and dried.

The membrane was placed in enhanced chemifluorescence (ECF)-solution and incubated for 30 seconds, before photographing it by using a Storm Bluescreen Storm 860 Scanner (Molecular Dynamics). The migration lengths of the marker proteins, measured from the start of the resolving gel, were plotted on the x-axis of a graph paper. The log of the marker protein sizes were plotted on the y-axis, and a standard curve was calculated. The unknown molecular weights were then determined. Subsequently, the quantities of the different protein products were estimated. The fluorescing intensity is directly related to the amount of protein present. Therefore, the protein quantity was estimated by compare the intensity of the desired bands to that of the background bands by using the ImageQuant 5.2 software (Amersham Biosciences).

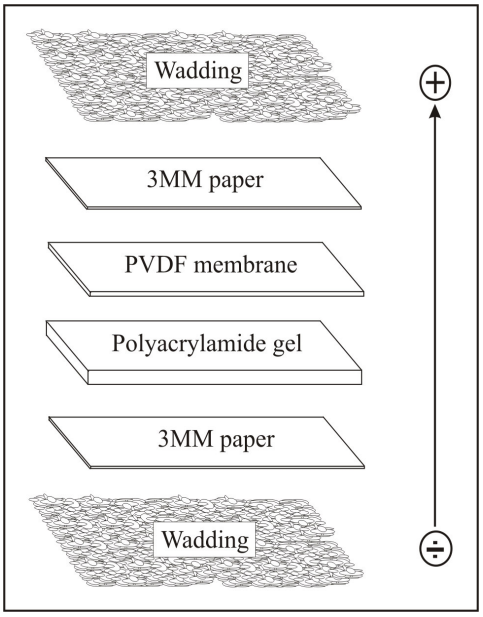

Figure 2-2 Western blotting transfer-unit assembly.

2.3.4 Immunocytochemistry

Immunocytochemistry was used to study the intracellular localization of TCF11. The proteins were visualized through immunofluorescence, using a fluorescein isothiocyanate (FITC) coupled antibody specific for a TCF11/Nrf1-recognizing antibody. The UV-excitable Hoechst 33342 nuclei stain was added to simplify the localization of the cells, and the images were acquired by means of epifluorescence and confocal microscopy.

2.3.4.1 Cell fixation and labelling

Cells growing on 10 mm^2 cover slips were transfected, followed by 24-30 hours of incubation.

The cover slips with the adhered transfected cells were washed twice for 2 minutes in PBS. The cells were then fixed in 200 µl 4% paraformaldehyde for 7 minute at RT, followed by two washes in PBS and three washes for 5 minutes in washing buffer (PBS, 1% BSA, 0.1% NP40, 0.001% NaAzide). The cells were then labelled with the primary antibody (1:1000 rabbit α-Nrf1 (C-19) [Santa Cruz Biotechnology] or 1:10000 rabbit α-TCF11 (53) in washing

buffer) ON at 4°C. Following three washes in washing buffer, the cells were labelled with the secondary antibody (1:330 Swine-anti rabbit FITC conjugate [DAKO] in washing buffer) for 60 minutes at RT, and then washed twice in washing buffer, once in PBS and finally rinsed off in MQ-water. The cover slips were mounted with the cell-side down in 5 µl of mounting solution (10% MOWOIL [Calbiochem]) containing 1 µg/ml Hoechst 33342 (Molecular Probes) for chromosome staining.

2.3.4.2 Epifluorescence microscopy

Images were acquired by means of a Leitz DM RXE microscope stand (Leica) equipped with a 40x/0.70 oil immersion objective and an F-view digital camera (Soft Imaging System). A UV-filter was used for the detection of hoechst, while a blue filter was used to detect FITC. Images were acquired and processed using the Analysis software (Soft Imaging System).

2.3.4.3 Digital Confocal Laser-Scanning Microscopy

Images were acquired with a Leica upright microscope stand equipped with a 100x/1.25 oil immersion objective, a TCS-SP digital scanning head (Leica) and a He/Ne/Ar laser lined at 488 nm for FITC excitation. To obtain a specific signal, the emition light was filtered through a 514-540 nm band pass. The z-position of the laser was adjusted to the nucleic centre of the cell, and the sample was scanned using the Leica confocal software.

3 Results

Preliminary studies in our group have indicated that high expression of TCF11 has a toxic effect on mammalian cells in culture. In addition, over-expression makes detailed cell-localization of the proteins difficult. To circumvent this problem, TCF11 could be put under the control of a weak promoter, either by inserting TCF11 into a vector containing a weak promoter, or by replacing a strong promoter with a weak promoter in an existing TCF11 construct. Both methods were attempted. In addition, low expressing plasmids containing Nrf1, a naturally occurring isoform of TCF11 lacking 30 aa's, was also constructed.

3.1 pRL-TK based TCF11/Nrf1 expressing plasmids

The initial idea in constructing low TCF11 and Nrf1 expression vectors was by cloning TCF11/Nrf1 into a pRL-TK vector. This vector contains the herpes simplex virus thymidine kinase (HSV-TK) promoter, which provides low to moderate levels of expression. By excising the *Renilla* luciferase (R*luc)* gene within the pRL-TK vector and inserting the TCF11 or Nrf1 genes, the resulting plasmids should express low levels of TCF11 or Nrf1.

3.1.1 Construction of pTK, pTK-TCF11 and pTK-Nrf1

The pRL-TK, pcTCF11 and pcNrf1 plasmids were transformed into *E.coli* cells (section 2.2.9) and plasmid DNA was isolated in midipreps from ON cultures (section 2.2.1). The plasmids were used to make the pTK-based constructs displayed in table 3-1.

Table 3-1 pTK-based constructs containing TCF11/Nrf1

Name	Description
pTK	pRL-TK vector with the R*luc*-gene excised.
pTK-TCF11	pTK with TCF11 inserted.
pTK-Nrf1	pTK with Nrf1 inserted.

The pTK construct was constructed by digesting the pRL-TK vector with *Xba* I and *Nru* I restriction enzymes (section 2.2.4), thus removing the R*luc*-gene from the vector and leaving compatible sticky ends. The opened vector was isolated and purified using preparative agarose gel separation (section 2.2.2), followed by re-ligation of the vector (section 2.2.6). Following transformation, twenty colonies were mini-prepared and analyzed by restriction analysis with *Hind* III. The restriction analysis gel of one positive clone of each plasmid is shown in figure 3-1. The cleaved pRL-TK vector displayed a ~4kb fragment (Fig. 3-1, lane 1), while the cleaved pTK vector revealed ~3.1kb fragment (Fig. 3-1, lane 2). Since the R*luc* gene is 0.9kb in size, the size difference between the two vectors demonstrates that the removal of R*luc* was successful.

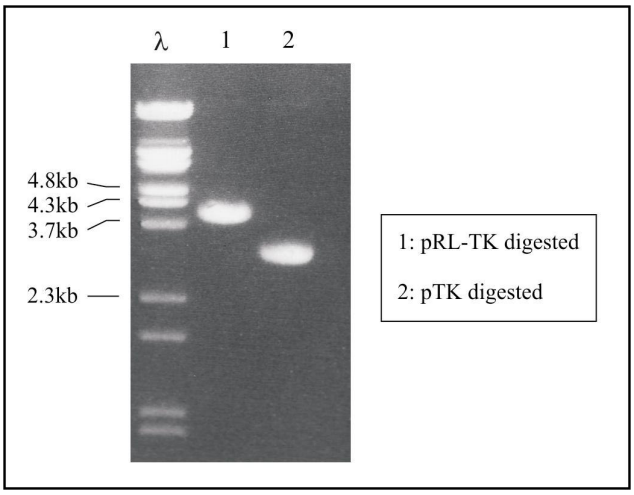

Figure 3-1Restriction analysis of pRL-TK and pTK. Lane 1 contains the pRL-TK vector digested with *Hind* III, while lane 2 contains pTK digested with *Hind* III.

The pTK plasmid was midi-prepared (section 2.2.1).

The construction of the pTK-TCF11 and pTK-Nrf1 plasmids was completed in several steps. The TCF11 and Nrf1 genes were isolated from the pcTCF11 and pcNrf1 plasmids through enzyme digestion and ligated with the pTK construct, as shown in figure 3-2.

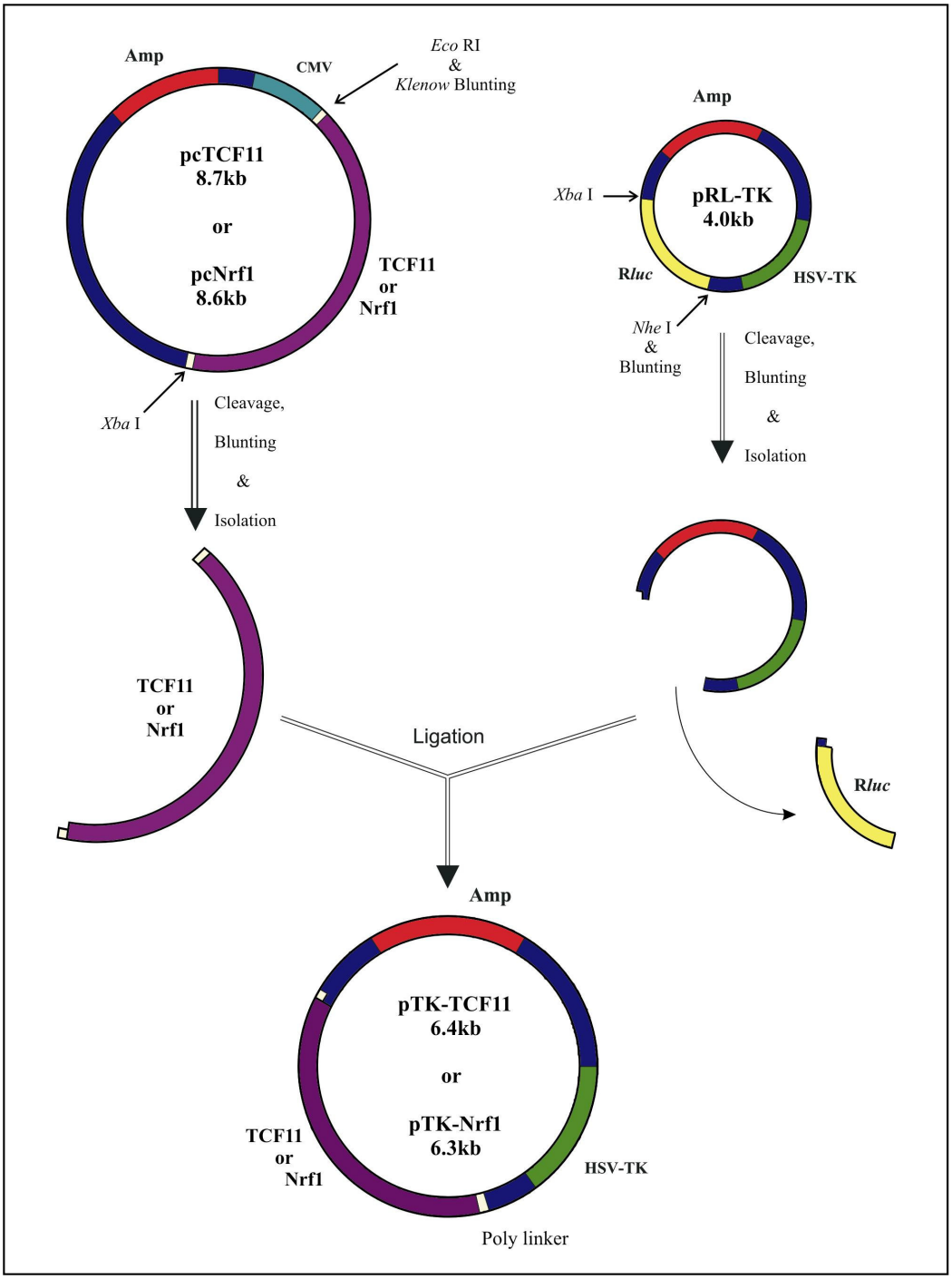

Figure 3-2 Construction of low expression plasmids by cloning TCF11/Nrf1 into pRL-TK vector : The TCF11/Nrf1 was isolated from the pcTCF11/pcNrf1 plasmids through *Eco* RI digestion and blunting with *Klenow* (section 2.2.7) , followed by digestion with *Xba* I. The R*luc* gene was excised from the pRL-TK vector through *Nhe* I digestion, blunting with *Klenow*, followed by digestion with *Xba* I. The opened pRL-TK vector and isolated TCF11/Nrf1 genes was ligated to form the pTK-TCF11/pTK-Nrf1 plasmids. The pTK vector was constructed by excising the R*luc* gene from the pRL-TK vector through *Xba* I and *Nru* I digestion, followed by re-ligation of the vector (not shown). The constructs were transiently transfected into COS-1 cells for further studies.

The ligated products were transformed and twenty colonies were mini-prepared and examined through restriction analysis with *Xba* I and *Psh* AI cleavage. The restriction analysis gel of one positive clone of each plasmid is shown in figure 3-3. Two fragments with sizes ~4kb and ~2.3kb were seen for the digested pTK-Nrf1 plasmid (Fig. 3-3, lane 4), two fragments with sizes ~4kb and ~2.4kb for the digested pTK-TCF11 plasmid (Fig. 3-3, lane 6) and one ~4kb fragment for the digested pRL-TK plasmid (Fig. 3-3, lane 2). This is compliant with the expected results since all three plasmids have one *Xba* I restriction site, while the *Psh* AI cleaves strictly in the TCF11 and Nrf1 containing plasmids, thus verifying that the cloning was successful.

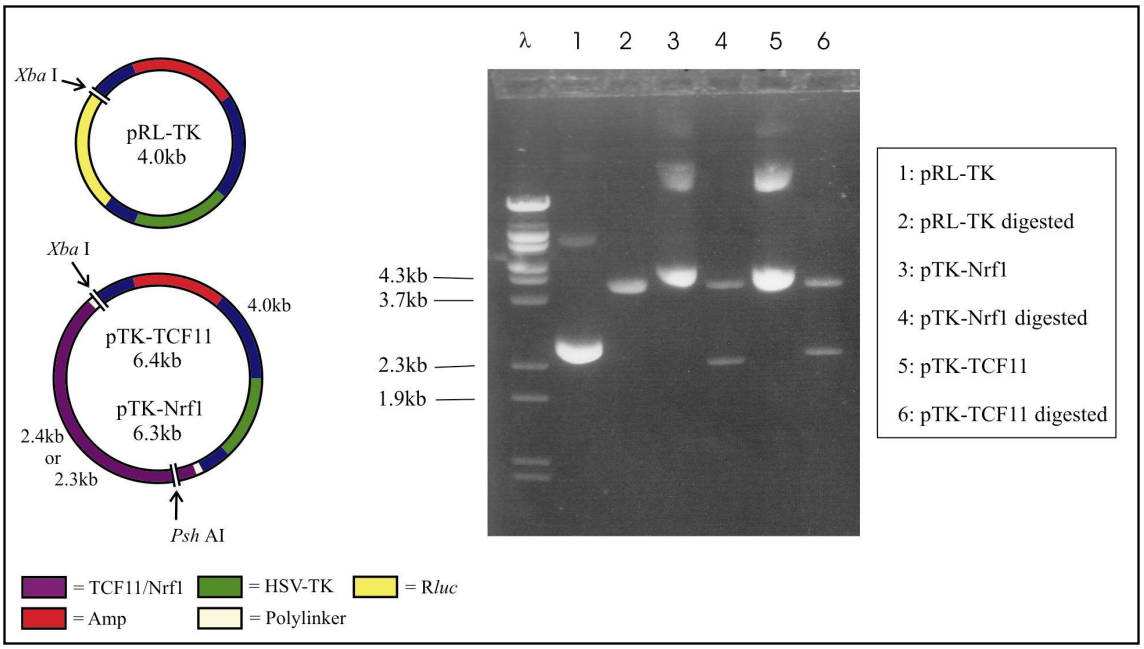

Figure 3-3 Restriction analysis of pRL-TK, pTK-TCF11 and pTK-Nrf1 plasmids. The pRL-TK (lane 2), pTK-Nrf1 (lane 4) and pTK-TCF11 (lane 6) constructs were digested with *Xba* I and *Psh* AI restriction enzymes. Lanes 1, 3 and 5 contain undigested plasmids. The plasmid maps show the restriction sites for both enzymes.

The constructs were midi-prepared and the identity was further confirmed by DNA-sequencing (section 2.2.11).

3.1.2 Transactivation assay of pTK-TCF11 and pTK-Nrf1

The transactivating ability of the pTK-based TCF11/Nrf1-containing constructs was analyzed by means of luciferase activity assays. The constructs were transiently co-transfected into COS-1 cells (section 2.3.1) with the 3.2PBGDLuc reporter plasmid, and the luciferase activity was measured (section 2.3.2) (Fig. 3-4). The experiment showed that the pTK-TCF11 plasmid (Fig. 3-4, bar 2), and the pTK-Nrf1 plasmid (Fig. 3-4, bar 3), gave a 0.6 and 0.3 fold reduction in activity, respectively, when compared to the empty pTK vector (Fig. 3-4, bar 1).

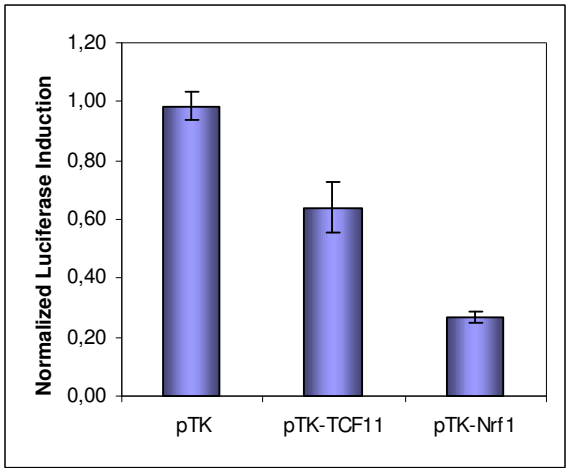

Figure 3-4 Luciferase induction by pTK-constructs transiently transfected in COS-1 cells: 0.2µg of the 3.2PBGDLuc reporter was co-transfected with 0.8 µg of either empty pTK vector (bar 1), or one of the TCF11/Nrf1 constructs (bars 2 and 3). The luciferase activity was normalized (section 2.3.2.2) and is relative to the activity given with the empty vector. The induction shown is the average of 3 experiments, and the *error bars* reflect the standard deviation of the mean value.

To investigate the ability of the pTK vector to activate luciferase transcription independently of the TCF11/Nrf1 proteins, COS-1 cells were transfected with an increasing amount of pTK to observe the variations in luciferase activity (Fig. 3-5). The co-transfection showed that an increase of transfected pTK induced an exponential increase of luciferase activity (Fig. 3-5, bars 1-3). The transfection of 2.0 µg of empty pTK vector (Fig. 3-5, bar 3) induced a 7.5 fold increase in luciferase activity compared to the activity of the empty pcDNA3 vector of equal transfectional amount (Fig. 3-5, bar 4). The 3.2PBGDLuc reporter by itself (Fig. 3-5, bar 5) did not induce any significant luciferase activity.

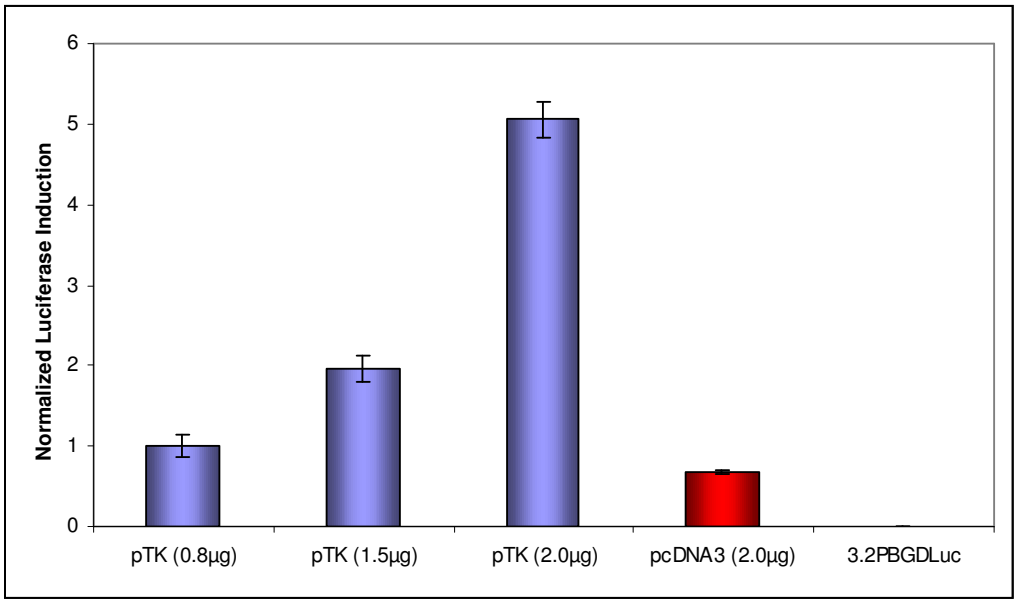

Figure 3-5 Luciferase induction in COS-1 cells transiently transfected with various amounts of pTK: 0.2 µg of the 3.2PBGDLuc reporter was co-transfected with 0.8, 1.5, or 2.0 µg of the pTK vector (bars 1, 2 and 3), or 2.0 µg of the pcDNA3 vector (bar 4). In addition, transfection with the reporter alone was also included (bar 5). The luciferase activity was normalized and is relative to the activity given with the 0.8 µg of pTK transfection (bar 1). The induction shown is the average of 3 experiments, and the *error bars* reflect the standard deviation of the mean value.

To investigate if the activation of luciferase transcription is NF-E2 site dependent, the pTK-TCF11 plasmid was transfected in the presence of normal 3.2PBGDLuc, but also with two reporters with mutations in the NF-E2 binding site. The first mutant reporter plasmid, 3.2PBGDLuc Mut.1, contains a 1-base mutation outside the AP-1 binding site, while the second reporter plasmid, 3.2PBGDLuc Mut.2, contains a 4-base mutation located within the AP-1 binding site (Fig. 3-6).

Co-transfection of the pTK-TCF11 plasmid and 3.2PBGDLuc Mut.1 reporter (Fig. 3-7, bar 3) showed an equivalent induction of luciferase activity to that of the pTK vector and 3.2PBGDLuc reporter co-transfection (Fig. 3-7, bar 1), and therefore a higher activity compared to the pTK-TCF11 and 3.2PBGDLuc co-transfection. The pTK-TCF11 and 3.2PBGDLuc Mut.2 co-transfection (Fig. 3-7, bar 4) showed a lower induction of luciferase activity compared to the pTK vector and 3.2PBGDLuc reporter co-transfection (Fig. 3-7, bar 1), but gave a similar luciferase induction to that of the pTK-TCF11 and 3.2PBGDLuc co-transfection (Fig. 3-7, bar 2).

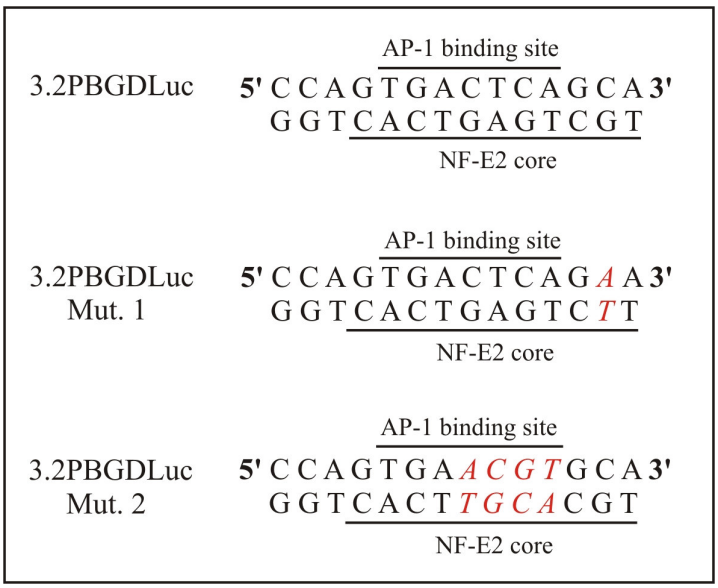

Figure 3-6 NF-E2 core sequence of 3.2BGDLuc, 3.2PBGDLuc Mut.1 and 3.2PBGDLuc Mut.2 reporter plasmids. The sequences represent the AP-1 binding site within the NF-E2 core binding site from the three reporter plasmids. The mutated regions are shown in red.

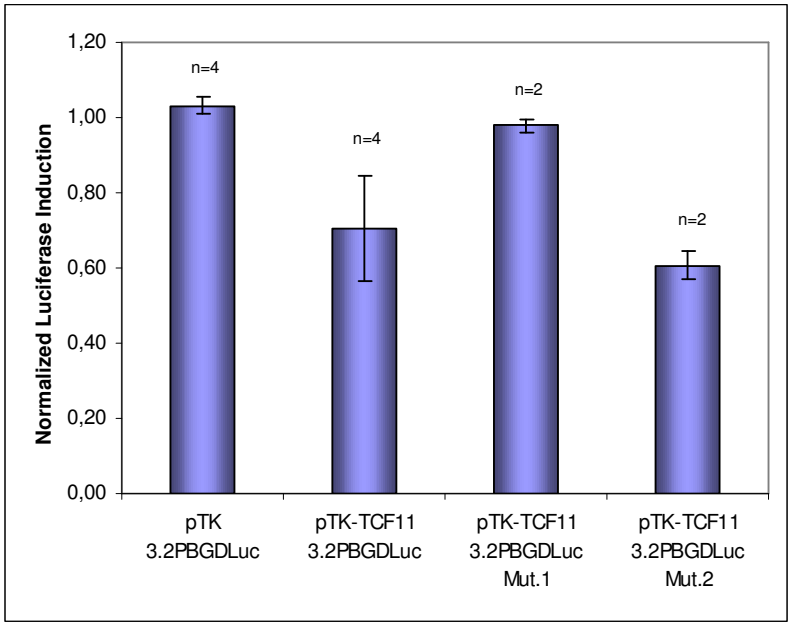

Figure 3-7 Luciferase induction in COS-1 cells transiently co-transfected with pTK-TCF11 and mutated reporter plasmids: 0.8 µg of pTK-TCF11 was co-transfected with 0.2µg of each the normal 3.2PBGDLuc reporter (bar 2) and the two mutated forms of the reporter plasmid, 3.2PBGDLuc Mut.1 and 3.2PBGDLuc Mut.2 (bars 3 and 4, respectively). In addition, 0.8µg of the empty pTK vector was co-transfected with 0.2 µg of the 3.2PBGDLuc reporter plasmid (bar 1). The luciferase activity was normalized and is relative to the activity given with the pTK vector (bar 1). The induction shown is the average over a number (n) of experiments, and the *error bars* reflect the standard deviation of the mean value.

3.1.3 Investigation of possible transcription regulator motifs

To investigate if the reporter induction was due to the presence of other potential transcriptional regulator binding sites, or motifs, the pcDNA3, pRL-TK and reporter vector were analyzed using the software program *Motif*. The search gave four different motifs that were located both in the pRL-TK and reporter vector, but absent in the pcDNA3 vector. These motifs bind the LyF-1, TCF3, HNF-3β and Brn-2 transcriptional regulators and were located at various sites in the vectors. Although none of the motifs were found in or near the PBGD promoter, the TCF3, HNF-3β and Brn-2 binding sites were all located within the luciferase gene.

3.2 pcDNA3 and HSV-TK based TCF11/Nrf1 expressing plasmids

Inserting the TCF11/Nrf1 genes into the pRL-TK vector created constructs that gave an unexpected result seeing that reporter induction occurred in the absence of TCF11 and Nrf1. Therefore, a different approach was devised. The weak promoter HSV-TK would now be inserted into the pre-made pcDNA3, pcTCF11 and pcNrf1 constructs, replacing the strong promoter CMV. This should transform the plasmids into lower expressing constructs.

3.2.1 Construction of pcDNA3-TK, pcTCF11-TK and pcNrf1-TK

To create the pcDNA3-based low expression constructs, the HSV-TK promoter was to be isolated from the pRL-TK vector. Two parallel approaches were attempted; the first was based on PCR amplification, whereas the second was based on enzymatic digestion. The latter proved to be the quicker method and was therefore chosen.

The isolation of HSV-TK and excision of CMV promoter from the pcDNA3 constructs was completed in several steps of digestion and purification, following ligation of the fragments as displayed in figure 3-8.

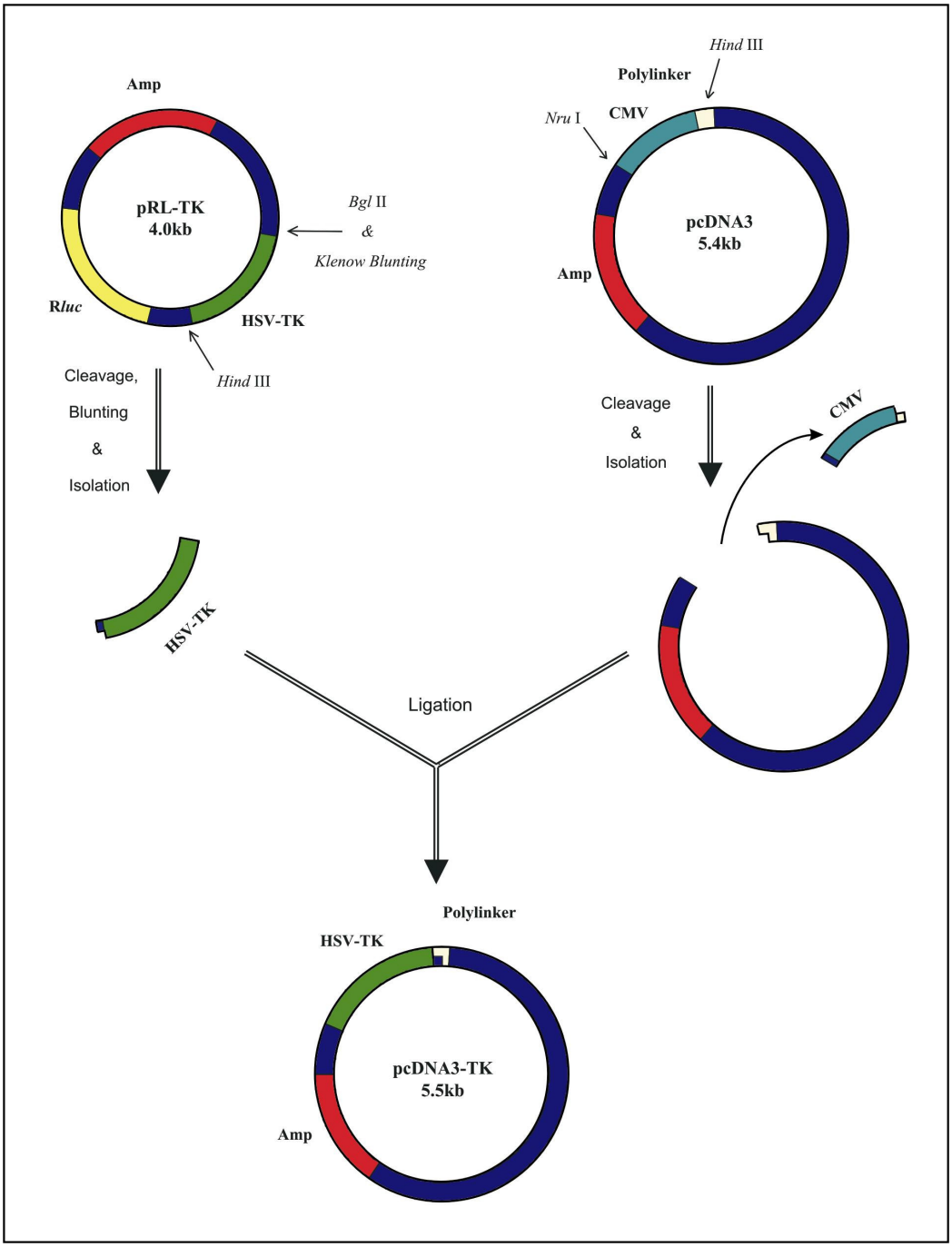

Figure 3-8 Construction of low expression plasmids by cloning HSV-TK into pcDNA3 : The HSV-TK promoter was isolated from the pRL-TK vector through *Bgl* II digestion and blunting with *Klenow*, followed by digestion with *Hind* III. The CMV promoter was excised from the pcDNA3-vector through *Nru* I and *Hind* III digestion. The opened pcDNA3 vector and isolated HSV-TK promoter was ligated to form the pcDNA3-TK construct. The pcTCF11-TK and pcNrf1-TK plasmids were constructed in the same manner. The constructs were transiently transfected into COS-1 cells for further studied.

The obtained ligated products were transformed, and twenty colonies were mini-prepared and analyzed through two separate restriction analysis cleaving with *Sac* II and *Sma* I. The restriction analysis gel of one positive clone of each plasmid is presented in figure 3-9. The plasmids digested with *Sac* II displayed only one fragment as expected (Fig. 3-9, lanes 2, 5, 8), since the enzyme only cleaves in the inserted HSV-TK promoter, thereby linearizing the plasmids. Digested pcDNA3-TK (Fig. 3-9, lane 2) showed a ~5.5kb fragment, while the digested pcTCF11-TK (Fig. 3-9, lane 5) and pcNrf1-TK (Fig. 3-9, lane 8) displayed fragments of sizes ~8.8kb and ~8.7kb, respectively, which were the expected sizes/fragments.

The plasmids cleaved with the *Sma* I enzyme (Fig. 3-9, lanes 3, 6 and 9) showed multiple fragments. Digested pcDNA3-TK (Fig. 3-9, lane 3) showed two fragments of sizes ~1.7kb and ~3.8kb, digested pcTCF11-TK (Fig. 3-9, lane 6) showed three fragments of sizes ~1.4kb, ~3.7kb and ~3.8kb, and the digested pcNrf1-TK (Fig. 3-9, lane 9) showed three fragments of sizes ~1.4kb, ~3.6kb and ~3.8kb. This was compliant with the expected result given that *Sma* I cleaves in the pcDNA3 vector, in the HSV-TK promoter, and in the TCF11/Nrf1 genes as displayed by the plasmid maps in figure 3-9.

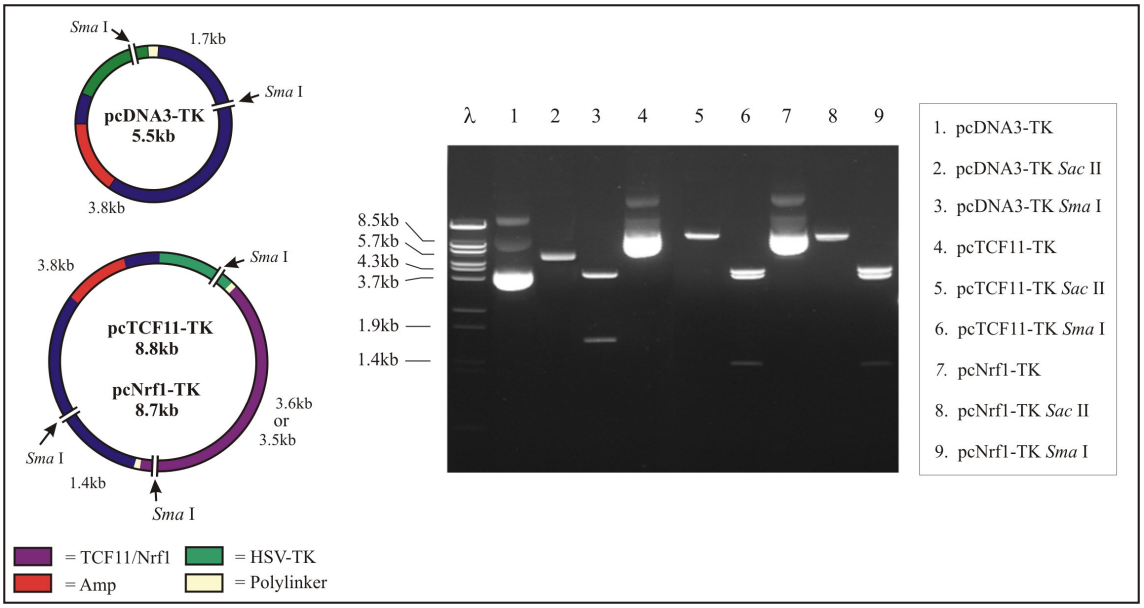

Figure 3-9 Restriction analysis of pcDNA3TK, pcTCF11-TK and pcNrf1-TK: Plasmids were cleaved with *Sac* II (lanes 2, 5 and 8) and *Sma* I (lanes 3, 6 and 9) restriction enzymes. Lanes 1, 4, and 7 contain undigested plasmids. The plasmid maps show the restriction sites for the *Sma* I enzyme.

The pcDNA3-TK, pcTCF11-TK and pcNrf1-TK plasmids were midi-prepared.

3.2.2 Transactivation assay of pcTCF11-TK and pcNrf1-TK

Prior to investigating the transactivating ability of the pcTCF11-TK and pcNrf1-TK constructs, the pcDNA3-TK vector was transfected in various amounts to see if it activated luciferase transcription in a similar manner to that of the pTK vector. The transfections did not reveal any significant variation in the induction of luciferase activity (Fig. 3-10).

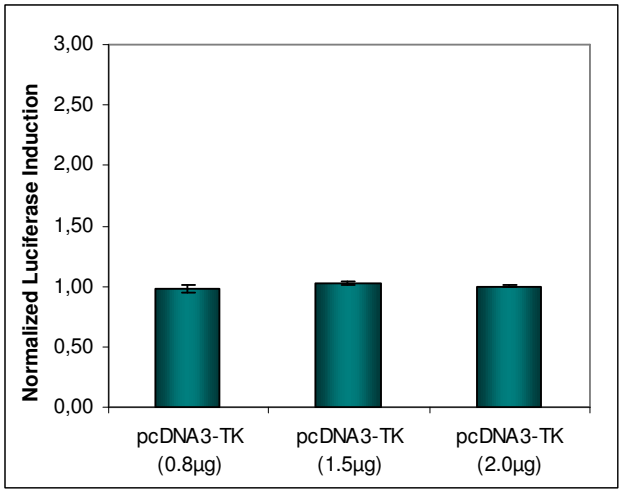

Figure 3-10 Luciferase induction in COS-1 cells transiently transfected with pcDNA3-TK: 0.2µg of the 3.2PBGDLuc reporter was co-transfected with 0.8, 1.5 or 2.0µg of the pcDNA3-TK construct (bars 1, 2 and 3, respectively). The luciferase activity was normalized and is relative to the activity given with the 0.8µg of pcDNA3-TK transfection (bar 1). The induction shown is the average of 3 experiments, and the *error bars* reflect the standard deviation of the mean value.

To investigate the transactivating ability of the pcTCF11-TK/pcNrf1-TK constructs, the plasmids were analyzed by means of a luciferase assay (Fig. 3-11). High expression constructs were included in order to compare the luciferase activities and observe if the low expression constructs produced a reduced level of TCF11/Nrf1 compared to that of the high expression constructs.

The high expression plasmid pcTCF11 (Fig. 3-11 A, bar 2) displayed a ten-fold increase in the induction of luciferase activity when compared to the induction of the low expression pcTCF11-TK plasmid (Fig. 3-11 B, bar 2). In addition, pcNrf1 (Fig. 3-11 A, bar 3) showed an average of 35% lower activity when compared to the activity of the pcTCF11 plasmid. The difference in activity between the pcTCF11-TK and the pcNrf1-TK plasmids (Fig. 3-11 B,

bars 2 and 3) could not be determined because the overall activities for the low expression plasmids were under the detection level of the assay.

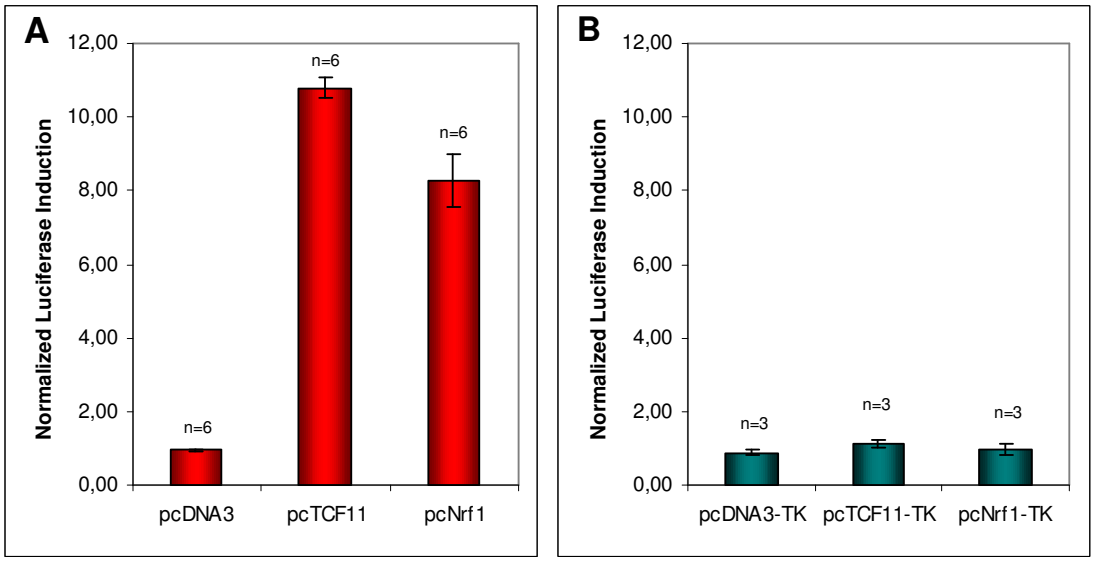

Figure 3-11 Luciferase induction in COS-1 cells transiently transfected with high and low TCF11/Nrf1 expressing plasmids: 0.2µg of 3.2PBGDLuc reporter was co-transfected with either (A) 0.8µg of the high expression TCF11/Nrf1 constructs (A, bars 2 and 3), including the empty pcDNA3 vector (A, bar 1) presented in red, or (B) 0.8 µg of the low expression TCF11/Nrf1constructs (B, bars 2 and 3), including the empty pcDNA3-TK vector (B, bar 1) presented in green. Luciferase activity was normalized and is relative to the activity given with the respective empty vectors. The induction shown is the average of a number of experiments (n), and the *error bars* reflect the standard deviation of the mean value.

To find the optimal transfection amount for the low expression plasmids, COS-1 cells were transfected with increasing amounts of expression DNA. The resulting luciferase assay (Fig. 3-12) showed that cells transfected with 2.0µg of pcTCF11-TK plasmid (Fig. 3-12, bar 4) induced a luciferase activity that was approximately three and a half times that of the background (Fig. 3-12, bar 1). Also noticeable for the 2.0µg transfections was the activity of the pcNrf1-TK (Fig. 3-12, bar 7), which was on average 33% lower than the activity of the pcTCF11-TK plasmid. For amounts lower than 2.0 µg, the luciferase activities were not significantly higher than that of the empty vector, while higher amounts gave results with great deviation (not shown).

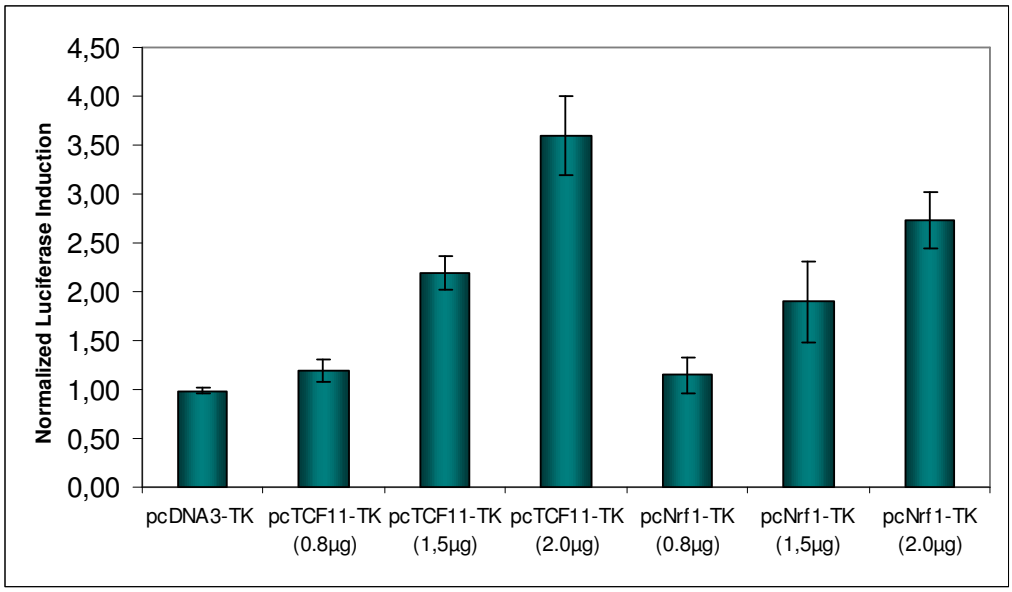

Figure 3-12 Luciferase induction in COS-1 cells transiently transfected with various amounts of low expressing TCF11/Nrf1 constructs: 0.2µg of the 3.2PBGDLuc reporter was co-transfected with either 0.8µg of the empty pcDNA3-TK vector (bar 1), or 0.8, 1.5 or 2.0µg of low expression TCF11/Nrf1 plasmids (bars 2 through 7). The total amount of transfected DNA was kept constant at 2.2µg by adding empty vector DNA (bars 2 through 7). Luciferase activity was normalized and is relative to the activity given with the empty vector. The induction shown is the average of 3 experiments, and the *error bars* reflect the standard deviation of the mean value.

3.2.3 Detection of TCF11/Nrf1 protein expression

The transactivating abilities of the high and low expression plasmids were investigated through luciferase activity assays. The studies displayed that the high expression plasmids gave a higher luciferase activity than that of the low expression plasmids. In addition, TCF11-containing plasmids gave higher activity compared to the Nrf1-containing plasmids, both for the low and the high expression plasmids.

To examine if the observed differences in luciferase activity were directly related to the expression of TCF11/Nrf1 protein in the cells, COS-1 cells transfected with high or low TCF11/Nrf1 expressing plasmids were analyzed through Western blot analysis (section 2.3.3). The Western blot is shown in figure 3-13. The pcTCF11 vector (Fig. 3-13, lane 2) displayed three translational products marked with (*); two of which were large proteins of ~160kDa and 140kDa, and a smaller protein of 65kDa. In addition, three possible processing products

are marked with (●) and are of sizes 45kDa, 32kDa, and 28kDa. The blot of pcNrf1 (Fig. 3-13, lane 3) displayed the same products, though the two large protein products appeared slightly smaller. There was no significant quantitative difference in the expression of TCF11 and Nrf1 proteins for the high expression vectors. However, the high expression vectors displayed a 2-3 times increase in expression of TCF11/Nrf1 proteins when compared to the low expression plasmids (Fig. 3-13, lanes 5 and 6). The protein expression displayed by the low expression constructs was too low to detect the putative difference between TCF11 and Nrf1. Also noticeable is the high background, represented by the bands present in the pcDNA3 and pcDNA3-TK control lanes (lanes 1 and 4, respectively).

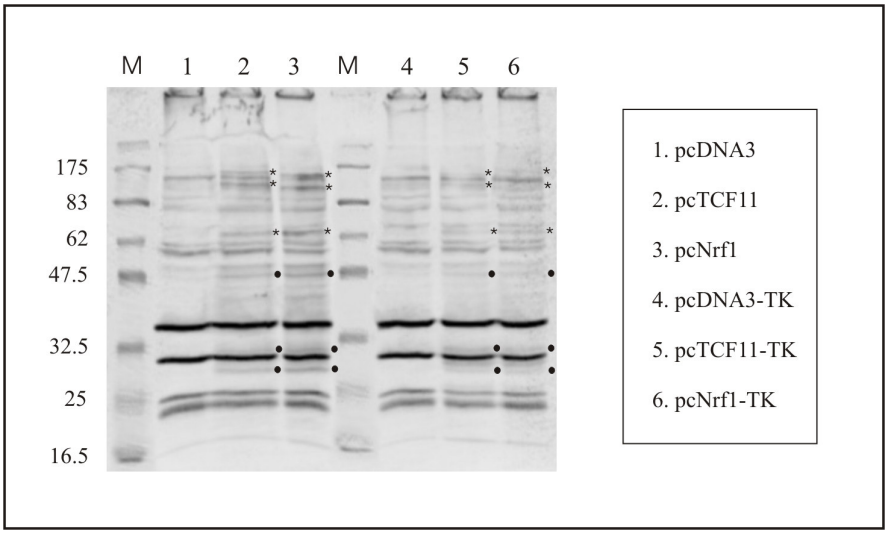

Figure 3-13 Detection of translational products using Western blot analysis: Whole cell extracts were prepared from COS-1 cells transiently transfected with 0.8µg of high and 2.0µg of low TCF11/Nrf1 expression constructs. After SDS-PAGE and transfer to a PVDF membrane, immunodetection with a 1:1000 dilution of the primary polyclonal antibody rabbit anti-Nrf1 and a 1:2000 dilution of the secondary antibody donkey anti-rabbit AP-conjugate revealed the different translational products (*), and possible processing products (●). The numbers on the left represent the size of the protein marker (M) in kDa. Extracts of cells transfected with pcDNA3 and pcDNA3-TK show background staining with this antibody. Expression from the transfected constructs is seen in addition to this background pattern.

3.2.4 Intracellular localization of TCF11 and Nrf1 proteins

To determine the intracellular localization of the TCF11 and Nrf1 proteins, high expression and low expression plasmids were transfected into COS-1 cells and prepared for immunocytochemistry (section 2.3.4) through cell fixation and labeling (section 2.3.4.1). Images were acquired through epifluorescence microscopy (section 2.3.4.2).

The epifluorescence microscopy images (Fig. 3-14) displayed high background staining, represented by the images of the pcDNA3-transfected control cells (Fig. 3-14, A). This complicated the intracellular localization of the TCF11 protein because it was difficult to distinguish the TCF11 from the background in the transfected cells expressing TCF11 (represented by Fig. 3-14 B [←]).

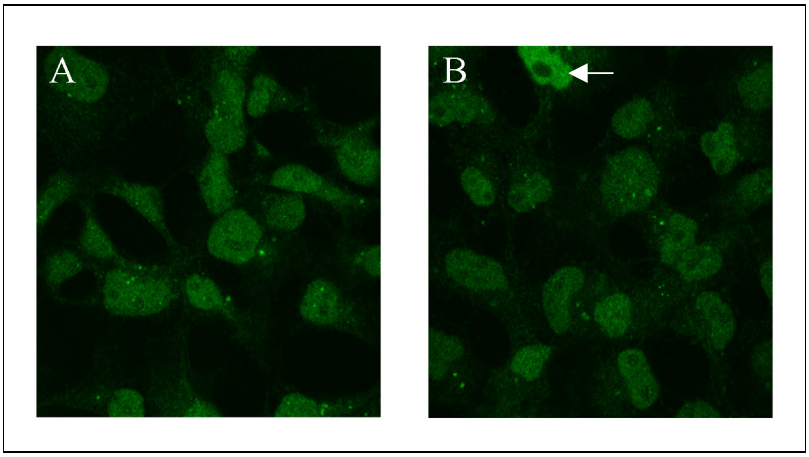

Figure 3-14 Epifluorescence microscopy studies of transiently transfected COS-1 cells: COS-1 cells were transiently transfected with 0.8 µg of high and low TCF11/Nrf1 expressing plasmids. Cells were fixed and stained with 1:1000 dilution of the primary Ab rabbit α-Nrf1, followed by 1:330 dilution of the fluorescence isothiocyanate (FITC) conjugated secondary Ab anti-rabbit IgG. Images were acquired with an epifluorescence microscope using a blue filter to detect FITC. The images displayed are pcDNA3 (A) and pcTCF11 (B) transfected cells, while the remaining images are not shown. Transfected cells are marked with (←). Images of cells transfected with pcDNA3 show background staining with this antibody.

The high background staining was caused by low specificity of the primary Ab since pcTCF11 transfected COS-1 cells stained directly with the secondary Ab did not reveal any background staining (not shown). A different primary antibody, the α-TCF11 (53), was

acquired and used along with the secondary Ab to label pcTCF11 transfected COS-1 cells. To find the optimal Ab dilution, the transfected cells were stained with a variety of Ab concentrations.

The images acquired for the Ab optimalization test (Fig. 3-15) displayed that COS-1 cells stained with a 1:10000 dilution of primary Ab and a 1:330 dilution of secondary Ab (panel C) produced the strongest signal with the least amount of background. Higher dilutions of the primary antibody were attempted, but did not give detectable signals (not shown).

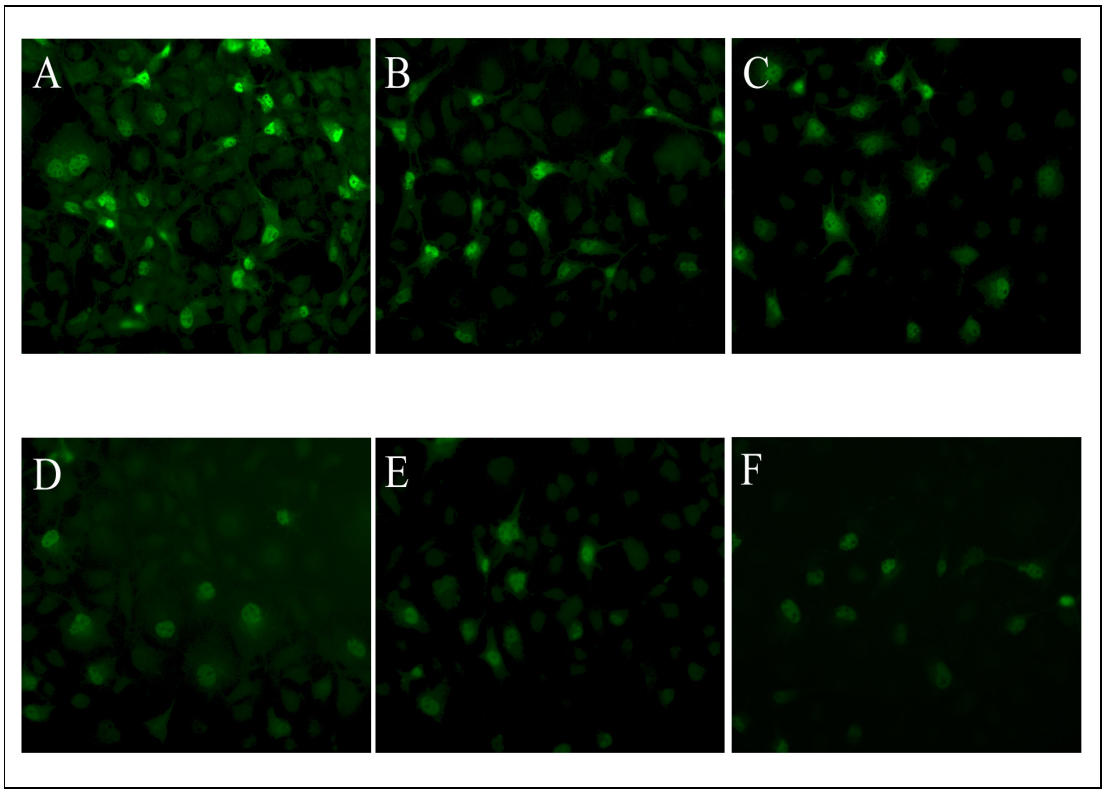

Figure 3-15 Epifluorescence microscopy studies of Ab-optimalization study: COS-1 cells were transiently transfected with 0.8 µg of pcTCF11 plasmid. Cells were fixed and stained with various dilutions of the primary Ab rabbit α-TCF11, followed by various dilutions of the fluorescence isothiocyanate (FITC) conjugated secondary Ab anti-rabbit IgG. Panels A-C displays cells stained with 1:2000, 1:5000 and 1:10000 of the 1ºAb along with 1:330 of the 2ºAb, respectively, while cells in panels D-F were stained with 1:2000, 1:5000 and 1:10000 of 1ºAb, along with 1:2000 of 2ºAb, respectively. Images were acquired with an epifluorescence microscope using a blue filter to detect FITC.

Epifluorescence microscopy images of the high and low TCF11/Nrf1 expressing plasmids by using the optimized dilution of the antibodies are shown in figure 3-16. The images of the high expression plasmids (Fig. 3-16, panes A and B) showed that TCF11 was located throughout the cells transfected with the pcTCF11 plasmid (Fig. 3-16, panes A1-A3). Similarly, the pcNrf1-transfection localized Nrf1 throughout the cell as well, (Fig. 3-16, panes B1-B3), however there were smaller quantities in the cytoplasm. The images of the low expression plasmids (Fig. 3-16, panes C and D) gave the same indication with respect to localization, but this could not be said with certainty due to the weak FITC fluorescence. On the other hand, the differences in intensity showed that there were reduced amounts of TCF11/Nrf1 in the low expression constructs compared to the high expression constructs.

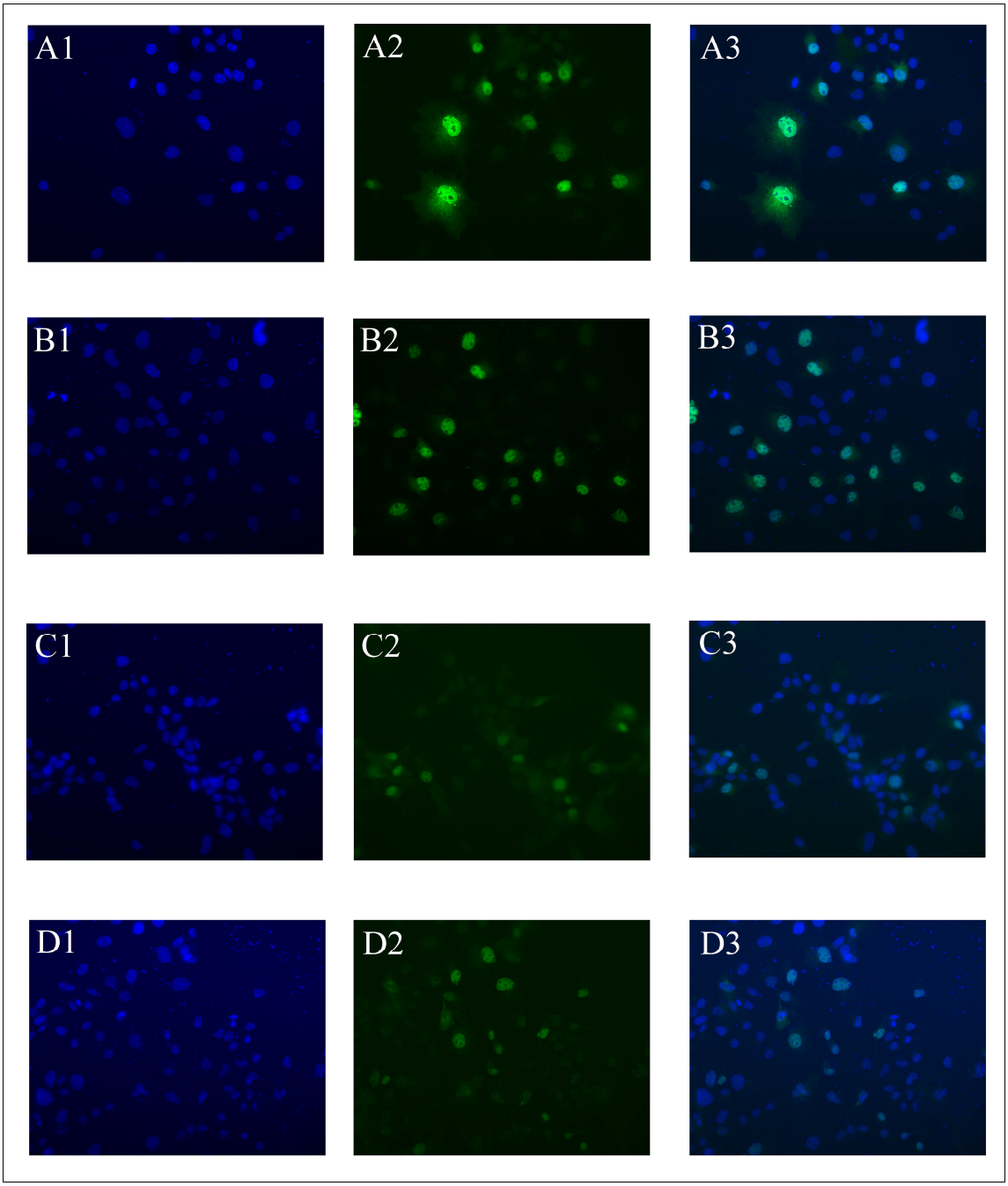

Figure 3-16 Epifluoresecence microscopy images of COS-1 cells transfected with pcTCF11, pcTCF11-TK, pcNrf1 and pcNrf1-TK: COS-1 cells were transiently transfected with 0.8 µg of (A) pcTCF11 and (B) pcNrf1 plasmids, and 2.0 µg of (C) pcTCF11-TK and (D) pcNrf1-TK plasmids. Cells were fixed and stained with 1:10000 dilution of the primary Ab rabbit α-TCF11, followed by 1:330 dilution of the fluorescence isothiocyanate (FITC) conjugated secondary Ab anti-rabbit IgG. The cells were subsequently stained with 1 µg/ml dilution of Hoechst 33342. Images were acquired with an epifluorescence microscope using a 40x/0.70 oil immersion objective, an F-view digital camera (Soft Imaging System), and a blue filter and UV-filter to detect FITC and Hoechst 33342, respectively. Images A1-D1 show the cells stained with Hoechst, images A2-D2 display cells stained with FITC, while A3-D3 represent the merged images.

To obtain a more detailed picture of the intracellular localization of TCF11/Nrf1 for both the high and low expression plasmids, images of the transfected cells were acquired through confocal microscopy (section 2.3.4.3). The images acquired (Fig. 3-17) showed that TCF11 was located in both the nucleus and cytoplasm in the pcTCF11 (Fig. 3-17, pane B) and pcTCF11-TK (Fig. 3-17, E) transfected cells. Similarly, the pcNrf1 transfected cells (Fig. 3-17, C) displayed Nrf1 in the nucleus and cytoplasm. However, the cells transfected with pcNrf1-TK (Fig. 3-17, F) displayed strict nuclear localization of Nrf1. The images did not reveal additional compartmentalization for TCF11 or Nrf1, apart from their lacking in the nucleoli (←).

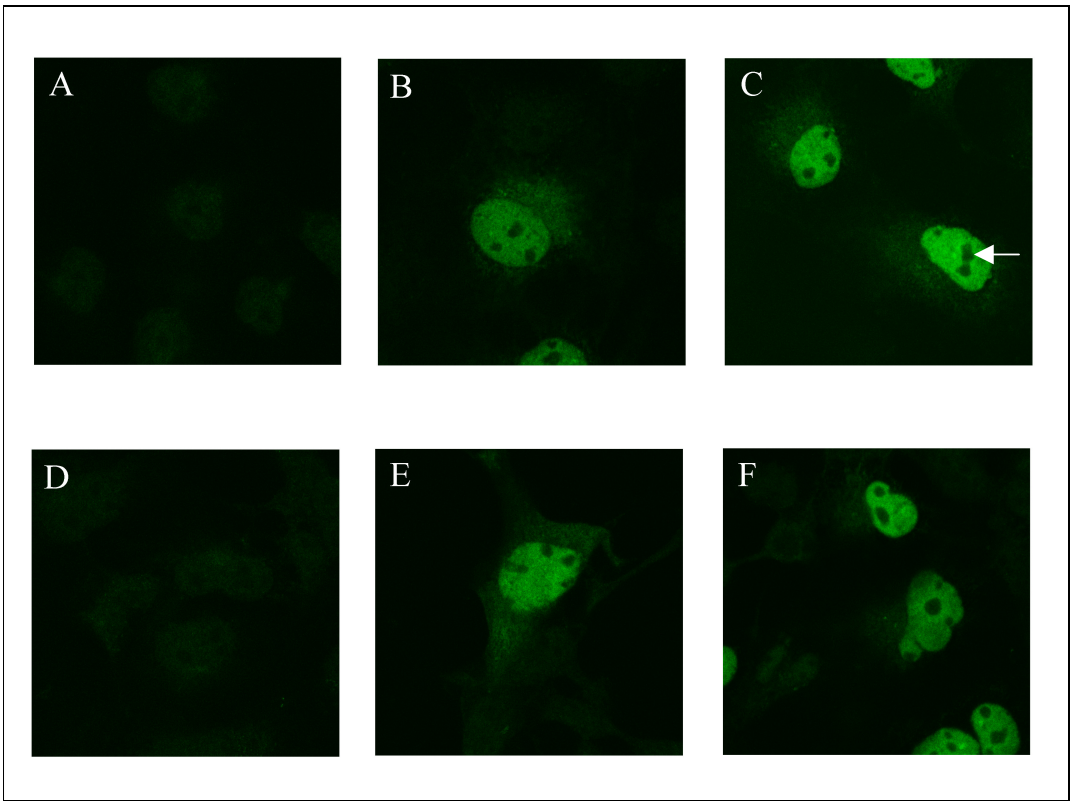

Figure 3-17 Confocal microscopy images of pcDNA3, pcDNA3-TK, pcTCF11, pcTCF11-TK, pcNrf1 and pcNrf1-TK: COS-1 cells were transiently transfected with 0.8 µg of (A) pcDNA3, (B) pcTCF11, and (C) pcNrf1 plasmids, and 2.0 µg of (D) pcDNA3-TK, (E) pcTCF11-TK, and (F) pcNrf1-TK plasmids. Cells were fixed and stained with 1:10000 dilution of the primary Ab rabbit α-TCF11, followed by 1:330 dilution of the fluorescence isothiocyanate (FITC) conjugated secondary Ab anti-rabbit IgG. Images were acquired with a digital confocal laser-scanning microscope using a 100x/1.25 oil immersion objective, a TCS-SP digital scanning head, and a He/Ne/Ar laser lined at 488 nm for FITC excitation. The images of the pcDNA3 (A) and pcDNA3-TK (D) transfected cells displays the background staining. One nucleolus is marked with (←).

4 Discussion

TCF11, along with its isoform Nrf1, belongs to the CNC-bZIP family of transcription factors. As mentioned earlier, the biological function of TCF11 and Nrf1 has yet to be determined, but their presence has been shown to be vital to embryonic development (60,61). Discovering the role of these transcription factors is therefore of great interest. The current vector system used to study TCF11 and Nrf1, however, has presented problems due to the high level of expression. One difficulty related to the over-expression of TCF11/Nrf1 is the observed cell-toxicity (personal communication, C. Husberg), which prevents long-term studies. In addition to toxicity, the over-expression has also made detailed intracellular localization studies of the proteins difficult, necessitating the need for a plasmid construct that could express lower levels of TCF11/Nrf1. The goal of this project was therefore to study the activity and localization of the TCF11 and Nrf1 transcription factors in a low expression vector system.

4.1 Transactivation assay of pTK, pTK-TCF11 and pTK-Nrf1

The pRL-TK vector, containing the weak promoter HSV-TK, was decided to be a suitable low expression vector. The TCF11/Nrf1 gene would be inserted, replacing the *Renilla* luciferase (R*luc*) gene, thus creating a weak TCF11/Nrf1 expressing plasmid. The excision of the R*luc* gene was not a necessity since firefly luciferase and R*luc* have different substrate specificities (92). However, it was completed in order to limit the size of the constructs, thereby simplifying the transfections. The creation of the pTK, pTK-TCF11, and pTK-Nrf1 constructs was carried out as shown in figures 3-1 and 3-3.

The transactivation measurements of the above constructs gave surprising results. COS-1 cells transiently transfected with the pTK-TCF11 and pTK-Nrf1 showed a 0.6 and 0.3 fold activation of luciferase, respectively, when compared to the pTK construct (Fig. 3-4). These results were unexpected given that pTK is a vector that, according to the technical specifications (93), should not express any products that may activate the reporter construct. However, cell lines contain numerous endogenous factors including transcription factors, co-factors and other accessory factors that are potential reporter activators (94,95). In addition, the gene expression of the reporter construct is controlled by the PBGD promoter, which has been shown to be regulated by several factors other than TCF11/Nrf1 (35).

Therefore, the reporter induction might be caused by the presence of endogenous factors in the COS-1 cells. However, the results (Fig. 3-5) showed that the transfection of the reporter construct alone did not give any noticeable luciferase activation, and the co-transfection with the empty pcDNA3 vector gave a very low activation of luciferase. In addition, the luciferase activity of pTK transfected cells increased considerably with increasing plasmid concentration and showed an activation that was 7.5 times higher compared to cells transfected with equivalent amounts of the pcDNA3 vector. Consequently, it appears as if the reporter induction is dependent on the presence of the pTK vector in addition to endogenous factors in the COS-1 cells.

The pRL-TK vector is intended for use as an internal control in transfection assays to correct for variations in transfection efficiency (93). In order for the internal control to be reliable, the vector should be unaffected by the different experimental conditions. However, several reports have shown that pRL-TK is sensitive to numerous transcription factors, hormones, and inhibitor molecules that regulate the R*luc* gene expression (94-99). The PPARα, HNF-4, COUP-TFI, and ARP-1 nuclear receptors all caused a repression of the pRL-TK luciferase expression when co-transfected in CV-1 cells (95). In contrast, the luciferase activity was elevated by the presence of dihydrotestosterone (DHT) in the same cell-line (94) and 12S E1A oncoprotein presence in Saos-2 cells (99). Hence, a possible reason for the observed high luciferase activity could be the result of the pRL-TK vector's sensitivity to regulatory factors. This possible explanation is presented in figure 4-1. COS-1 cells, as mentioned earlier, contain numerous endogenous factors that may induce or repress the reporter. However, in the absence of the pRL-TK vector, the reporter was not activated. This inhibition may be caused by inactivating factor(s) binding to activating factor(s), thereby preventing luciferase activation (Fig 4-1 A-1). Another possibility could be that inhibiting factors bind to repressor sites on the reporter, preventing activating factors from inducing the reporter (Fig 4-1 B-1). Thus, if the pTK vector contains motifs that titer out these inhibiting factors, its presence may result in an increase in luciferase activation through the release of the activating factors (Fig. 4-1 A-2), or the removal of the repressor factors (Fig. 4-1 B-2).

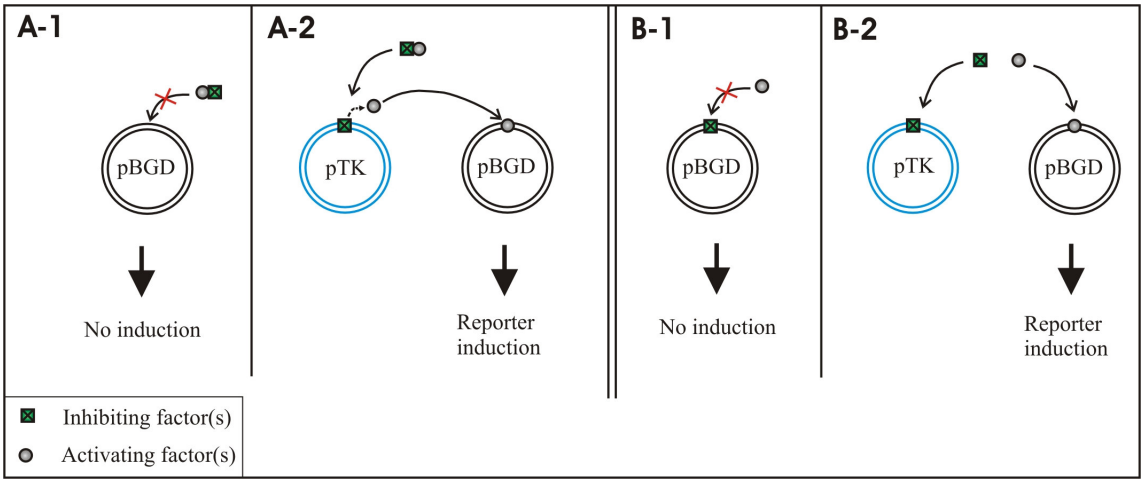

Figure 4-1 Theoretical models for the observed luciferase activation due to the presence of pTK. (A-1) Inhibiting and activating agents form a complex that cannot activate the pBGD (3.2PBGDLuc) reporter. (A-2) pTK binds the complex thereby releasing the activating factor, which subsequently induces the reporter. (B-1) Inhibiting factors bind to the reporter thereby preventing the induction by activating factors. (B-2) pTK binds and titers out the inhibiting factors, allowing for the activating factors to induce the reporter.

The observed elevated luciferase activity caused by an increasing pTK concentration (Fig. 3-5) supports the above mentioned theories. In these experiments a possible linear relationship between luciferase activity and pTK concentration could not be examined because the total DNA amount was not kept constant through the addition of an empty vector. This vector addition was excluded because different vectors have been seen to affect one another (100), which could have a detrimental effect on the experiment. This was also the reason why an internal control, which measures transfection efficiency, was excluded in favor of protein content normalization in combination with numerous experimental repeats. Although the latter method merely normalizes for sample loss during processing (101), transfections by means of FuGENE have previously been determined to be adequately stable (personal communication, C. Husberg).

One way to identify the endogenous inhibitory and activating factors that potentially caused the observed luciferase activity could be to establish possible target binding sites within the vectors. The observation that the presence of TCF11/Nrf1 had a reducing effect on luciferase activation (Fig. 3-4) suggested that the cause of the induction is somehow related to proteins binding to the NF-E2/AP-1 site. This was supported by the results obtained from the luciferase assays involving the co-transfection of normal and mutated 3.2PBGDLuc reporter constructs with the pTK-TCF11 construct (Fig. 3-7). A theoretical model explaining the

observed results is presented in figure 4-2. The reduced activation due to the presence of TCF11 may indicate that TCF11 has a stronger binding affinity to the NF-E2/AP-1 site, but at the same time causes lower luciferase activation compared to the proposed endogenous factors (Fig. 4-2 B). The co-transfection with pTK-TCF11 and 3.2PBGDLuc Mut.1 reporter showed an induction equivalent to the pTK and wild type reporter co-transfection. This mutation, which has been shown to prevent TCF11 from binding to the NF-E2 site (55), would still allow AP-1 binding factors to bind (Fig. 4-2 C). However, the pTK-TCF11 and 3.2PBGDLuc Mut.2 reporter co-transfection gave a luciferase activation that was reduced to that of the pTK-TCF11 and wild type reporter co-transfection. This mutation prevents not only TCF11, but also AP-1 binding factors, from binding to the NF-E2/AP-1 binding site (55) (Fig. 4-2 D). These results suggest that the presence of pTK makes AP-1 binding factors available to the AP-1 site in the promoter (Fig. 4-2 A).

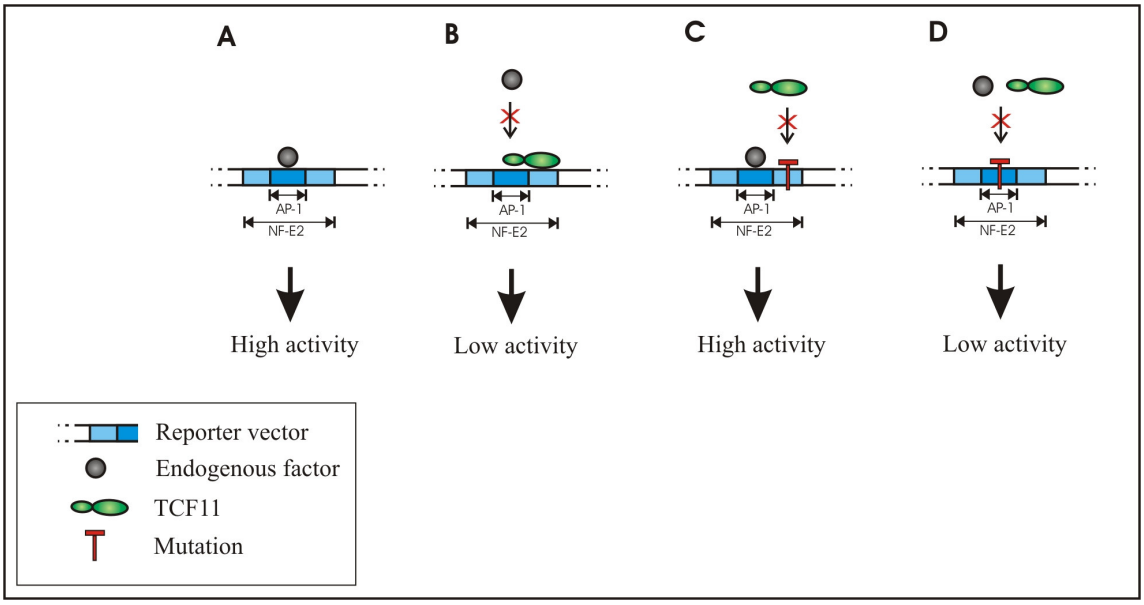

Figure 4-2 Theoretical model explaining the effects of the PBGD promoter mutation on luciferase activation. (A) In the absence of TCF11, the endogenous factor(s) bind to the AP-1 site and cause high luciferase activity. (B) In the presence of TCF11 the endogenous factor(s) cannot bind to the AP-1 site and the activity is reduced. (C) When the NF-E2 site is mutated, TCF11 cannot bind, allowing endogenous factors to bind to the AP-1 site and activate the reporter. (D) When the AP-1 site mutation, neither factors can bind, resulting in reduced activity.

However, even though the AP-1 site mutation resulted in decreased luciferase activation, it was still excessive compared to the activation caused by the presence of the pcDNA3 vector. This signified the existence of additional reporter activating and inhibiting factors in the COS-

1 cells, and consequently additional binding motifs. By analyzing the sequences of pcDNA3, pTK and 3.2PBGDLuc constructs using the software program *Motif* (http://motif.genome.ad.jp), four different binding motifs where found that existed exclusively in the reporter plasmid and pRL-TK vector. The motifs bind LyF-1, TCF3, HNF-3β and Brn-2 transcriptional regulators. None of the motifs were located in or near the PBGD promoter, however the TCF3, HNF-3β and Brn-2 are all located within the luciferase gene. This location may be of importance based on previous studies indicating the possibility that the regulation of the luciferase gene was dependent on interactions between nuclear receptors and the luciferase gene itself (95). If factors bound to the mentioned motifs have an inhibiting effect on the reporter, their binding to the motifs on the pTK may allow for reporter activation, supporting the theory presented in figure 4-1 B.

The mentioned motifs' importance in this luciferase assay is unknown. Nevertheless, their lacking in the inert pcDNA3 vector presents a possible explanation for the observed luciferase activity. Deleting or mutating the motifs in the pTK vector and observing the changes in luciferase activation could be an effective test to determine their importance. However, an important limitation in this approach is the presumed inability of the *Motif* software program to detect all possible motifs in the vectors. Furthermore, the models presented (Fig. 4-1 and 5-2) may represent an over-simplification of the actual mechanisms. There may be numerous protein-protein interactions that ultimately result in the inhibition/activation of the reporter plasmid, as appear to be the situation for COUP-TFI activation of the vHNF-1 promoter (102) and HIV 1 LTR promoter (103).

A possible strategy to avoid the observed pTK-caused induction, thereby enabling the use of the constructs, could be to utilize an alternative cell-line. Studies have shown that the activities of transcription factors are cell-line depend. The tk-CAT reporter containing the arrestin promoter was activated by the nuclear receptor ARP-1 in CV-1 cells, but unaffected in NG108 neuronal cells (104). In a different study, the COUP-TFI transcription factor induced a CAT reporter containing the mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase promoter in HepG2 cells, while the reporter activity was suppressed in R2C cells (105). However, due to the high sensitivity shown by pRL-TK in several cell lines, the success of this approach may have a high degree of uncertainty.

The reasons for the observed reporter induction by the presence of pTK may be numerous, and an interesting path to follow. However, the main objective of this thesis was to create and examine a plasmid expressing low levels of TCF11/Nrf1. Therefore, the investigation involving pTK was stopped at this point.

4.2 Transactivation assay of pcTCF11-TK and pcNrf1-TK

The alternative approach adopted due to the unwanted reporter induction, was to replace the strong promoter CMV in the pre-made pcDNA3 constructs with the weak promoter HSV-TK from the pRL-TK vector, thus transforming the plasmids into lower expressing constructs. The pcDNA3-TK, pcTCF11-TK and pcNrf1-TK constructs were created (Fig. 3-9).

To ensure that the high luciferase activity seen for the pTK transfections did not occur for the pcDNA3-TK vector, the transactivating ability of the latter was established. The results showed that the luciferase induction was low and did not increase as the plasmid concentration increased (Fig. 3-10). This indicates that the HSV-TK promoter was not directly involved in the high induction of the reporter plasmid caused by the presence of the pTK vector (section 4.1).

Subsequently, the transactivating abilities of the pcTCF11-TK and pcNrf1-TK plasmids were tested and compared to that of the high expression constructs pcTCF11 and pcNrf1 (Fig. 3-11). The pcTCF11-TK and pcNrf1-TK constructs caused a ten-fold reduced induction compared to the pcTCF11 and pcNrf1, respectively, and were therefore termed ”low expression constructs”. However, the luciferase activities observed for the low expression constructs were not within the detection limits of the assay. To confirm that the constructs induced the reporter, the low expression plasmid concentration was increased from 0.8µg to 2.0µg (Fig. 3-12), which gave a luciferase activation that was three and a half times that of the background for the pcTCF11-TK transfection. Furthermore, the epifluorescence images (Fig. 3-16) and the Western analysis (Fig. 3-13) supported the hypothesis that the observed decrease in luciferase activation for the low expression constructs were due to a lower protein concentration. The Western analysis revealed a two to three times lower expression of TCF11 and Nrf1, which is in accordance to the observed luciferase activation where the low expression construct gave a ~2.8 times reduced induction compared to the high expression constructs. The Western gel also revealed several translational and processing products that

have previously been identified by our group (50). The large proteins of 160kDa and 140kDa represent the long active form of TCF11/Nrf1 whereas the 65kDa product represents the shorter inactive form of the proteins (49). These processing products have not been studied extensively. However, since the antibody utilized in this study is designed to recognize the C-terminal end of TCF11/Nrf1, these products can be assumed to cover the C-terminal parts of the proteins. The observed high background staining could either indicate that the antibody showed low specificity, or high non-specific interaction. Unfortunately, the alternative antibody α-TCF11 that was used for the intracellular localization studies (Section 3.2.4) was not used because stocks were depleted.

4.2.1 Intracellular localization of TCF11 and Nrf1

Previous studies on intracellular localization indicated that TCF11 had both a cytoplasmic and a nuclear localization, while Nrf1 was only found in the nucleus. This was found to be due to the absence of a functional NES-signal in Nrf1 (50). However, additional localization details were difficult to obtain due to the high expression of TCF11/Nrf1. It was therefore interesting to compare the high expression to the low expression constructs to see if a reduced amount of TCF11/Nrf1 could reveal additional information about their intracellular localization.

The initial epifluorescence microscopy images did not reveal the intracellular localization of the proteins due to the excessive background shown by the commercial primary antibody (Fig. 3-14). A different primary antibody (Section 3.2.4) was therefore utilized following optimalization (Fig. 3-15). The subsequent images acquired by using epifluorescence microscopy (Fig. 3-16) supported the Western analysis results (Section 4.2), displaying a reduced level of expression of TCF11/Nrf1 for the low expression compared to the high expression constructs. The reduced level of TCF11/Nrf1 in the low expression construct cells, however, made intracellular localization difficult to determine from the acquired images. The images of the high expression constructs, on the other hand, revealed that both proteins were present in the cytoplasm and the nucleus, although the level of cytoplasmic Nrf1 were somewhat reduced. The subsequent confocal microscopy images (Fig. 3-17) gave similar results for the high expression constructs. However, the low expression constructs images displayed the presence of TCF11 in both the cytoplasm and the nucleus, while Nrf1 appeared to be strictly localized to the nucleus. This could indicate that the cytoplasmic Nrf1 seen in the high expression plasmid images could represent newly translated protein that had not yet

trafficked to the nucleus, as was shown by Alefantis *et al* (106) with the cytoplasmic localization of the NES-mutated protein Tax.

Additional compartmentalization of the proteins could not be detected, except for the absence of TCF11/Nrf1 in the nucleoli.

4.2.2 Differences in TCF11 and Nrf1 activity

Previous studies on the transactivating ability of Nrf1 and TCF11 have shown ambivalent results, with Nrf1 activity being similar to TCF11 in some cases (50), while displaying reduced activity compared to TCF11 in other studies (personal communication, E. Bjørgo). The results in this thesis supports the latter, with both the high and low Nrf1 expressing constructs displaying a reduction in luciferase activation when compared to that of TCF11 expressing constructs (Fig. 3-11 and 3-12). pcTCF11 and pcTCF11-TK appeared to give on average 34% higher luciferase activation compared to that of pcNrf1 and pcNrf1-TK, respectively. These are surprising results seeing that strict nuclear localization of Nrf1 (Section 4.2.1) would suggest an elevated activation compared to that of TCF11, due to increased availability to the target DNA. This could either indicate that the expression of Nrf1, or that its transactivating ability, was lower than that of TCF11. The Western analysis of parallel transfections did not reveal any significant deviation in the expression of the two proteins (Fig. 3-13). Furthermore, previous studies focusing on DNA binding ability did not present any significant differences between TCF11 and Nrf1 (50). This suggests that TCF11 has a higher transactivating ability compared to Nrf1.

One reason for the difference in the ability to transactivate may be due to the fact that 30 amino acid residues are lacking from the AD in Nrf1. Studies have shown that proteins with increasing deletions of the AD sequences display a gradual loss of activity (107,108). The partial deletion of the main AD in Nrf1 may therefore pose an explanation to the reduction in activity.

Another possible explanation for the elevated activity of TCF11 may be found in the regulating mechanism of nuclear export. The precise manner in which this mechanism is regulated has yet to be discovered for TCF11. However, one recognized way of regulating NES-dependent nuclear export is by means of phosphorylation (72,84,109-111).

Phosphorylation is predicted to cause conformational changes in the protein, thereby exposing NES and allowing for nuclear export (84,109,110). Studies on TCF11, on the other hand, have indicated that interference with several potent phosphorylation sites, including a serine rich region linked to the activation of TCF11, did not impede nuclear export (50). Still, there may be numerous alternative sites that are of importance, and studies have shown that activation-related phosphorylation of a protein can be separate from the phosphorylation necessary to stimulate NES-related nuclear export (90,110). Therefore, suggesting that phosphorylation may be a prerequisite for nuclear export of TCF11 to occur, it is plausible that this modulation will take place for the isoform Nrf1 despite the absence of NES. This is supported by studies on the Snail transcription regulator (72), Smad4 protein (83), and the Smad1 protein (112), which showed that NES-related phosphorylation of proteins occurred independently of whether or not the NES signal was functional or mutated. The same studies reported further that the transactivating abilities of the mutated proteins were reduced, like that observed with Nrf1. In the case of the Snail transcription regulator, this reduction was explained by suggesting that the phosphorylated mutated protein retained its ability to bind DNA, while the ability to regulate the reporter was reduced (72). This could explain the observed decrease in activation that was non-attributable to the level of Nrf1 expression. Since the activity of Nrf1 cannot be controlled by translocating out of the nucleus, like that seen for TCF11, phosphorylation may therefore act as a mechanism controlling the activity of Nrf1 by reducing its ability to activate the target promoters.

Notably, an increased reduction of activation due to the suggested phosphorylation could be expected. However, *in vivo*, with endogenous levels of Nrf1 in a balanced situation, the regulatory system will not encounter Nrf1-expression of such magnitude like that seen when using the strong CMV promoter, or even the weaker HSV-TK promoter to control the expression. As an example, the N-WASP expressed under the CMV promoter was estimated to be 20-fold higher than that of endogenous N-WASP (109). Therefore, natural small changes in the availability of Nrf1 may therefore be controlled more efficiently by the proposed phosphorylation.

4.3 Conclusions

The pRL-TK vector was deemed unreliable to be used as an expression vector for TCF11 because its presence appeared to indirectly cause activation of the pBGD reporter vector. The manner in which this activation occurred is unknown, but it may be due to its reported high level of sensitivity towards endogenous factors. pRL-TK may titer out inhibiting factors, thereby allowing activating factors to gain access to and activate the reporter construct. This high sensitivity displayed by pTK towards another vector and/or endogenous factors emphasizes the caution that must be taken when using internal control plasmids to measure transfection efficiencies.

The creation of pcDNA3-based constructs containing the HSV-TK promoter gave plasmids that expressed TCF11 and Nrf1 in lower amounts. These constructs may therefore elude the toxicity due to over-expression, allowing for prolonged studies of TCF11.

The low expression constructs allowed for easier verification of the nuclear detainment of Nrf1, however additional intracellular compartmentalization was not observed. In addition, the transactivating ability of TCF11 was shown to be higher than Nrf1 in both the high and low expression constructs.

4.4 Future Aspects

Initially, this thesis was meant to focus solely on constructing vectors that expressed reduced levels of TCF11 and Nrf1, and subsequently using these vectors to obtain information about the proteins' localization, activation, and dimerization abilities. However, due to unexpected results, the use of pRL-TK (internal controls) in luciferase activation measurements was also examined. The reason behind the reporter plasmid induction caused by the presence of the pTK vector was left unanswered. However, studying the cause could be of high relevance to the scientific community, given that pRL-TK is intended for use as an internal control. If the reason for the induction is found, one could either alter the vector, thereby averting the problem, or make people aware of the potential problem associated with using pRL-TK with a similar transfectional set-up as presented in this thesis.

The validity of the theory suggesting that the induction was caused by endogenous factors binding to pTK motifs could be investigated by deleting or mutating these motifs. If these sites did not prove to be of importance, a more extensive mutation/deletion study on pTK could be performed bearing in mind that the program *Motif* may omit motifs. Another option could be to co-transfect pTK and the reporter into alternative cell lines and subsequently observe if the variation of endogenous factors prevents the induction.

The mortality rate of cells with lower expression of TCF11/Nrf1 was not studied due to time limitations. It could therefore be interesting to stably transfect cells with the low expression constructs and observe the viability of the cells.

An interesting follow-up could also be to examine whether or not there exists a phosphorylation site that regulates both the nuclear export of TCF11 and activation of Nrf1. This could be revealed by extensively mutating or deleting potential phosphorylation sites in TCF11 and Nrf1, and subsequently observe the effects on the activity and localization of the proteins.

The low expression plasmids could also be utilized to study what effect the presence of dimerization partners like MafG have on both gene expression and the intracellular localization of TCF11 and Nrf1. Reversely, it could be interesting to see if the presence of

TCF11 and Nrf1 causes differences in the intracellular localization of the dimerization partners due to the presence or absence of NES. It may therefore be necessary to construct low expression vectors of the dimerization partners to simplify the localization procedure.

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